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GENETIC MANIPULATION OF THE PANCREAS:
CELL AND GENE THERAPY APPROACHES FOR TYPE 1 DIABETES

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EDUARD AYUSO LÓPEZ **FÀTIMA BOSCH I TUBERT**

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ABBREVIATIONS

AAV	Adeno-asociated virus
ABC	Avidin-biotin complex acid
Ad	Adenovirus
ADP	Adenosine-5'-diphosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine-5'-triphosphate
BAD	Pro-apoptotic protein
BM	Bone marrow
BMC	Bone marrow cells
bp	base pair
BSA	Bovine Serum Albumin
ß-gal	ß-galactosidase
cAMP	cyclic adenosine-5'-monophosphate
CAR	Coxsackie virus adenovirus receptor
cDNA	copied DNA
CAG	cytomegalovirus enhancer and chicken beta actin hybrid promoter
Ci	curie
CMV	cytomegalovirus
Con	control
cpm	counts per minute
DAB	3'3'-diaminobenzidine
DAG	diacylglycerol
dATP	desoxiadenosin-5'-triphosphate
DCCT	*diabetes control and complications trial*
dCTP	desoxicitidin-5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	Desoxiribonucleic acid
Dnase	deoxyribonuclease
dNTPs	2'-deoxynucleosides 5'-triphosphate
ds	double stranded
EDTA	ethylenediamine-tetraacetic acid
Fas	Fas, cell death receptor
FasL	Fas ligand
FBS	Fetal Bovine Serum
FG-Ad	First generation adenoviral vectors
FITC	*fuorecein isothiocyanate*
g	grams
GAD	Glutamic acid decarboxilase
GFP	Green fluorescein protein
GH	Growth hormone

GK	glucokinase
GLP-1	*glucagon-like peptide 1*
h	hour
hCMV	human cytomegalovirus
HC-Ad	High capacity adenoviral vectors
HD-Ad	Helper-dependent adenoviral vectors
HEK	Human Embryo Kidney
HIV	human immunodeficiency virus
HLA	Human leukocyte antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IFN-β	beta interferon
IFNβ/IGF-I	double transgenic mice RIP/IFNβ and RIP/IGF-I
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-IR	Insulin-like growth factor receptor-I
IL	interleukine
IM	Intramuscular
iNOS	inducible nitric oxide sinthase
Ip	intraperitoneal
IP3	inositol triphosfphate
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
IU	infection units of adenoviral particles
Kb	Kilobase
KDa	kiloDalton
KO	knock-out
M	molar (mol/l)
MAPK	mitogen-activated protein kinase
MCS	Multiple Cloning Site
mg	milligrams
MHC	major histocompatibility complex
min	minute/s
ml	millilitre
MLD	*multiple low dosis*
mM	millimolar
mRNA	RNA messenger
NO	Nitric oxide
NOD	*non obese diabetic*
o/n	over night
OD	optical density
^{32}P	Phosphor 32
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDX-1	pancreatic duodenal homeobox gene

PI-3K	phosphatidyl inositol 3 kinase
PKB	protein kinase B
p p	Physical particles
r.t.	room temperature
RIP/ hIFNβ	Rat insulin promoter-I– cDNA human IFNβ
RIP/ IGF-I	Rat insulin promoter-I– cDNA murine IGF-I
RIP-I	Rat insulin promoter-I
RIP-II	Rat insulin promoter-II
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate
sec	seconds
SEM	standard error from the mean
Ss	single stranded
SSC	sodium chloride and sodium citrate solution
SMA	smooth muscle actin
STZ	streptozotocine (2-deoxi-2-(3-metil-3-nitrosourea)1-Ddlucopyranose
Tg	transgenic
TRITC	texas red isothiocyanate
UV	Ultraviolet
vol	volum
vg	vector genomes
WPRE	Woodchuck post-transcriptional regulatory element
x g	gravity unit

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Thesis Contents

I. SUMMARY

Type 1 diabetes is characterized by progressive destruction of pancreatic β-cells, resulting in insulin deficiency and hyperglycemia. Insulin replacement therapy allows diabetic patients to lead active lives, but this therapy is imperfect and does not prevent development of severe secondary complications. Transplantation of pancreatic tissue or islets has been performed successfully in a limited numbers of patients. However, the shortage of donors is a primary obstacle that prevents this treatment from becoming more widespread. Therefore, many efforts have been focused on differentiating embryonic or adult stem cells into ß-cells. Bone marrow cells (BMCs) are an important source of easily procurable adult stem cells and have been proposed as an alternative source of ß-cells. Insulin-like growth factor-I (IGF-I) participates in skeletal muscle regeneration and enhances the recruitment of BMCs at the sites of muscle injury. In addition, IGF-I expression in ß-cells of diabetic transgenic mice regenerates pancreatic ß-cell mass. Therefore one of the objectives of this study was to investigate whether IGF-I expression in ß-cells could increase BMC recruitment and differentiation into ß-cells under steady-state conditions or after STZ treatment. To this end, BMCs from ß-actin/GFP transgenic donor mice were transplanted into IGF-I transgenic mice. Our experiments have demonstrated that IGF-I overexpression or STZ-induced pancreatic damage were not sufficient to recruit and differentiate GFP-labelled BMCs into ß-cells *in vivo*, indicating that these cells did not contribute to the endocrine pancreas regeneration observed in IGF-I transgenic mice. These data suggest that replication of pre-existing ß-cells and/or differentiation from non-BMC precursors is the most likely mechanism for IGF-I-mediated regeneration.

Diabetes mellitus has long been targeted, as yet unsuccessfully, as being curable with gene therapy. Recovery from type 1 diabetes requires ß-cell regeneration. One approach to do so is by genetically engineering the endocrine pancreas *in vivo* to express factors that induce ß-cell replication and neogenesis and counteract the immune response. However, the pancreas is difficult to manipulate and pancreatitis is a serious concern, which has made effective gene transfer to this organ elusive. Thus, new approaches for gene delivery to the pancreas *in vivo* are required. In this study we have

examined different viral vectors and routes of administration in rodents and also in large animals, to determine the most efficient method to deliver exogenous genes to the pancreas. First, we observed that pancreatic ß-cells were efficiently transduced to express ß-galactosidase after systemic injection of adenoviral vectors in mice with clamped hepatic circulation. This was true both for first generation as well as for helper-dependent adenoviral vectors. In addition to adenoviruses, we have compared the ability of AAV vectors to transduce the pancreas *in vivo* after intravascular, intraperitoneal or intraductal delivery, being the last the most efficient route of administration.

Like the human pancreas, the canine pancreas is compact, with similar vascularization and lobular structure. It is therefore a suitable model in which to assess gene transfer strategies. Here we examined the ability of adenoviral vectors to transfer genes into the pancreas of dogs in which pancreatic circulation has been clamped. Adenoviruses carrying the ß-galactosidase (ß-gal) gene were injected into the pancreatic-duodenal vein and the clamp was released 10 min later. These dogs showed ß-gal-positive cells throughout the pancreas, with no evidence of pancreatic damage. ß-gal was expressed mainly in acinar cells, but also in ducts and islets. ß-gal expression in the exocrine pancreas of a diabetic dog was also found to be similar to that observed in healthy dogs.

Thus, the methodology described herein may be used to transfer genes of interest to murine and canine pancreas *in vivo*, both for the study of islet biology and to develop new gene therapy approaches for diabetes mellitus and other pancreatic disorders.

II. INTRODUCTION

1. STRUCTURE AND FUNCTION OF THE PANCREAS.

The embryonic pancreas in vertebrates forms from a dorsal and ventral protrusion of the primitive gut epithelium. These two pancreatic buds grow, branch and ultimately fuse to form the definitive pancreas. The exocrine pancreas consists of acinar cells that produce and secrete a variety of digestive enzymes such as proteases, lipases and nucleases (Fig. 1). Another component of the exocrine pancreas is the highly branched ductal epithelium, which transports the digestive enzymes and bicarbonate ions to the intestine, where they contribute to the digestion of food. The endocrine pancreas constitutes about 2-3% of the total gland, and consists of four cell types α-, β-, δ-, and pancreatic-polypeptide cells that produce the hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively (Fig. 1). Endocrine cells form clusters, called islets of Langerhans, and they are scattered irregularly throughout the exocrine parenchyma, most densely in the tail region. The ß-cells form a core that is surrounded by the other cell types. Although the bulk of the pancreatic endocrine tissue is organized as islets, single extrainsular endocrine cells can occasionally be found (Rahier *et al.*, 1981). Of the endocrine cells, ~60–80% are insulin-producing ß-cells, 15–20% are glucagon-producing α-cells, 5–10% are somatostatin-producing δ-cells and <2% are pancreatic-polypeptide-producing cells (Fig. 1).

Insulin is released from ß-cells in response to increased levels of blood sugar after food intake; this is a signal for the target tissues (liver, muscle and fat) to take up glucose. In addition, insulin inhibits glucose production in the liver. By contrast, glucagon secretion is stimulated at low blood-sugar levels. Glucagon promotes glycogenolysis and gluconeogenesis as the level of glucose in the blood decreases.

Somatostatin and pancreatic polypeptide exert inhibitory effects on both pancreatic endocrine and exocrine secretion.

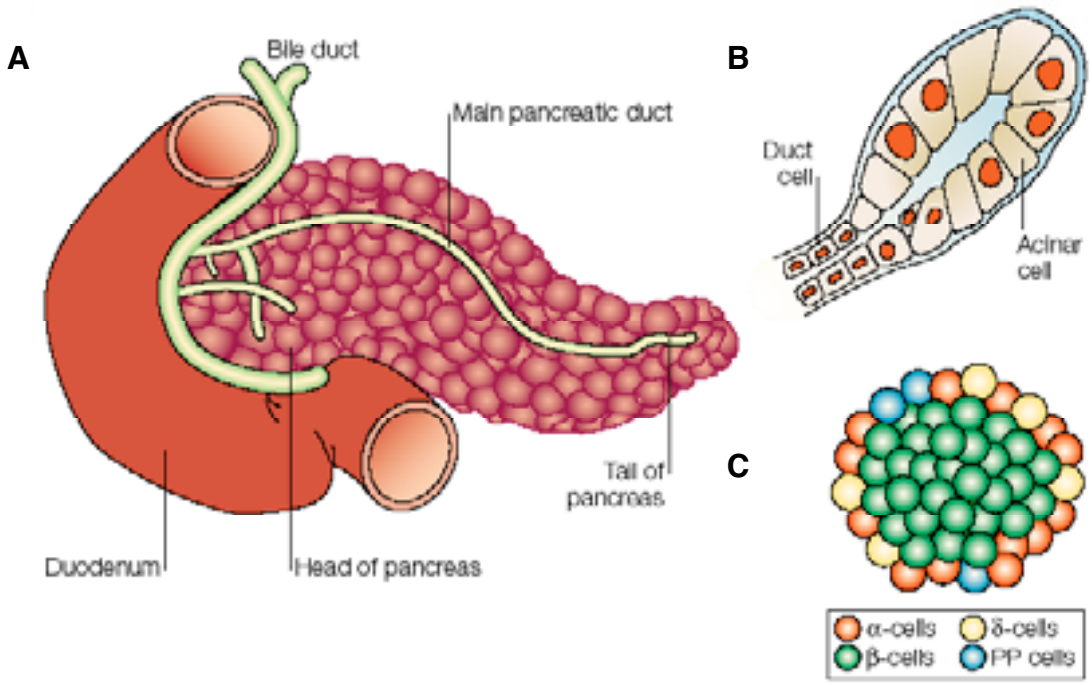


Figure 1. The pancreas is a mixed exocrine and endocrine organ. a) The mature pancreas is adjacent to the duodenum, the most anterior part of the small intestine. **b)** The function of the exocrine pancreas is to supply the gut with digestive enzymes; these are produced and secreted by acinar cells and subsequently transported to the intestine via the pancreatic ductal system. **c)** Endocrine pancreas consists of four hormone producing cell types α-, β-, δ-, and pancreatic polypeptide (PP) cells.

There are several differences between the rodent pancreas and large animals and humans. In rodents, the pancreas is a diffuse organ, whereas in large animal and humans it is compact. In the dog, it has the classic V-shape, consisting of two lobes (right and left) that emerge from the pancreatic body, surrounded by a delicate capsule of connective tissue. Septa from the capsule divide the pancreas into lobules, delimited by connective tissue, which produce a nodular surface with irregular crenate margins. Between the lobules, connective tissue surrounds the larger ducts, blood vessels, and nerve fibers. Branches from the celiac and the cranial mesenteric arteries supply blood to the pancreas. The pancreatic branches of the splenic artery irrigate the left lobe,

whereas the right lobe is irrigated by the cranial and caudal pancreaticoduodenal arteries (branches from the gastroduodenal artery (cranial) and cranial mesenteric artery (caudal), respectively). Anastomoses are common in pancreatic circulation. Veins are parallel to the arteries and eventually drain into the portal vein.

Although the islets constitute only 2-3% of the total pancreas mass, they receive around 20% of the blood flow (Iismaa *et al.*, 2000). The pattern of blood flow through the islets appears to be specifically organized. Blood flows from ß to α and then to δ cells (Bonner weir S., 1991) and presumably carries metabolic and hormonal information from the ß-cells to the mantle. The pancreatic islets are innervated by parasympatic cholinergic, sympathic adrenergic and various peptidergic nerves. The pancreas receives its extrinsic innervation from the celiac plexus.

2. TYPE 1 DIABETES.

Diabetes mellitus is the most prevalent metabolic disorder, and is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Pickup, J.C. and Williams, G., 1994). Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

Diabetes mellitus is usually classified as type 1 or type 2 diabetes. Type 1 diabetes, previously encompassed by the terms insulin-dependent diabetes, which

affects 5 to 10% of the diabetic population, typically appears during youth and results from the autoimmune destruction of the islet ß-cells within the pancreas (Atkinson and Maclaren, 1994). Markers of the immune destruction of the ß-cell include islet cell autoantibodies (ICAs), autoantobodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2ß (Baekkeskov *et al.*, 1982; Atkinson *et al.*, 1986; Christie *et al.*, 1992; Kaufman *et al.*, 1992; Schmidli *et al.*, 1994; Schott *et al.*, 1994; Myers *et al.*, 1995; Lan *et al.*, 1996; Lu *et al.*, 1996). One and usually more of these autoantibodies are present in 85-90% of individuals when fasting hyperglycemia is initially detected. Also, the disease has strong HLA associations, with linkage to DQA and B genes, and it is influenced by the DRB genes (Cantor *et al.*, 1995; Huang *et al.*, 1996). These HLA-DR/DQ alleles can be either predisposing or protective.

In this form of diabetes, the rate of ß-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults) (Zimmet *et al.*, 1994).

Autoimmune destruction of ß-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Although patients are rarely obese when they present this type of diabetes, the presence of obesity is not incompatible with the diagnosis. These patients are also prone to other immune disorders such as Grave's disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, and pernicious anemia.

Some forms of type 1 diabetes have no known etiologies. Some of these patients have permanent insulinopenia and are rare prone to ketoacidosis, but have no evidence of autoimmunity. Although only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African and Asian origin. Individuals with this

form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited, lacks immunological evidence for ß-cell autoimmunity, and is not HLA associated. There is an absolute, yet periodic, requirement for insulin replacement therapy in affected patients (Banerji and Lebovitz, 1989).

3. CURRENT THERAPY FOR TYPE 1 DIABETES.

3.1. Insulin replacement.

Conventional treatment of type 1 diabetes patients consists in daily subcutaneous injections of recombinat insulin, which attempt to mimic the physiological levels of the hormone. However, glycemia is not always properly regulated, and chronic hyperglycemia leads to severe microvascular, macrovascular and neurological complications (Pickup, J.C. and Williams, G 1994). The Diabetes Control and Complications Trial Group compared the effect of intensive therapy, administered either with an external pump or by three or more daily insulin injections, with conventional treatment, consisting in one or two daily insulin injections, in the ability to prevent secondary complications (The Diabetes Control and Complications Trial Research Group, 1993). Intensive therapy of patients with type 1 diabetes delays the onset and slows the progression of clinically important retinopathy, nephropathy, and neuropathy, by a range of 35 to more than 70 percent. In contrast, the risk of severe hypoglycemia was three times higher with intensive therapy.

3.2. Pancreas or islet transplantation.

3.2.1. Pancreas transplantation.

Pancreas transplantation was first described in 1967 (Kelly *et al.*, 1967), but initial pancreas graft and patient survival rates were dismal. A variety of factors, including advances in surgical techniques, immunosuppression, graft preservation techniques, methods of diagnosis and treatment of rejection, and management of common posttransplant complications, have led to significant improvements in graft and patient survival. As a result, the total number of pancreas transplant procedures reported to United Network of Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) continued to increase, a total of 18,843 from December 1966 to October 2002, with most (13,951) performed in the United States (Gruessner and Sutherland, 2002).

Pancreas transplantation is actually a group of procedures, each with particular characteristics that, in some cases, may have different, immediate, and possibly long-term complications and outcomes. In most cases, pancreas transplantation is performed in the setting of type 1 diabetes with end-stage renal disease (ESRD). The frequency of diabetes as the etiology of ESRD has doubled over the last decade so that diabetes, both type 1 and type 2, is now the most common cause of new ESRD (Collins *et al.*, 2005). Because the longevity of patients with type 1 diabetes has increased, more individuals are at risk of developing ESRD, increasing the number of type 1 diabetes patients also eligible for combined or simultaneous pancreas-kidney transplant. Kidney transplant markedly improves patient survival in the diabetic ESRD patient compared with dialysis, especially when performed early (Ojo *et al.*, 1998; Wolfe *et al.*, 1999; Meier-Kriesche *et al.*, 2000; Mange *et al.*, 2001). Therefore, the impact of adding a pancreas

graft, as with simultaneous pancreas-kidney transplant, should improve patient survival to that of kidney transplant alone.

There are three main types of pancreas transplantation (Larsen, 2004): 1) simultaneous pancreas-kidney transplant, in which the pancreas and kidney are transplanted from the same deceased donor; 2) pancreas after-kidney transplant, in which a cadaveric, or deceased, donor pancreas transplant is performed after a previous, and different, living or deceased donor kidney transplant; and 3) pancreas transplant alone for the patient with type 1 diabetes who usually has severe, frequent hypoglycemia, but adequate kidney function. Pancreas transplant alone and pancreas-after-kidney transplant candidates must have stable, adequate kidney function at the time of transplant, as both the transplant operation and immunosuppression can otherwise cause an immediate further decline of renal function. Immediate complications that can occur with all types of pancreas transplant include rejection, thrombosis, pancreatitis, and infection.

3.2.2. Islet transplantation.

Islet transplantation has been investigated as a treatment for type 1 diabetes mellitus in selected patients with inadequate glucose control despite insulin therapy. However, the hope that such an approach would result in long-term freedom from the need for exogenous insulin has failed to materialize in practice until the Edmonton protocol described by Shapiro and collaborators in 2000 (Shapiro *et al.*, 2000). Since 1990 to 2000, of the 267 allografts transplanted only 12.4 % have resulted in insulin independence for periods of more than one week, and only 8.2 % have done so for periods of more than one year. In the majority of these procedures, the regimen of immunosuppression consisted of antibody induction with an antilymphocyte globulin

combined with cyclosporine, azathioprine, and glucocorticoids (Brendel et. al 1999). However, for any type of transplantation procedure, a balance is sought between efficacy and toxicity. To address this problem, the Edmonton group used a glucocorticoid-free immunosuppressive protocol that included sirolimus, lowdose tacrolimus and a monoclonal antibody against the interleukin-2 receptor (daclizumab) for a trial of islet transplantation alone in patients with type 1 diabetes (Shapiro *et al.*, 2000). All patients quickly attained sustained insulin independence after transplantation. All recipients required islets from two donor pancreases, and one required a third transplant from two donors to achieve sustained insulin independence (Shapiro *et al.*, 2000). A five-year follow up of the Edmonton group was reported recently (Ryan *et al.*, 2005). Sixty-five patients received an islet transplant in Edmonton as of 1 November 2004. Five subjects became insulin independent after one transplant. Fifty-two patients had two transplants, and 11 subjects had three transplants. In the completed patients, 5-year follow-up reveals that the majority (approximately 80%) have C-peptide present post-islet transplant, but only a minority (approximately 10%) maintain insulin independence. The median duration of insulin independence was 15 months. In addition, islet transplantation relieved glucose instability and problems with hypoglycemia (Ryan *et al.*, 2005). The results, though promising, still suggest the need for further progress in the availability of transplantable islets, improving islet engraftment, preserving islet function and reducing toxic immunosuppression. Therefore, the search for alternative sources of ß-cells and protection of these ß-cells upon transplantation are very active fields in diabetes research.

4. CELL THERAPY FOR TYPE 1 DIABETES

Diabetes mellitus has long been targeted, as yet unsuccessfully, as being curable with cell and/or gene therapy. The main hurdles have not only been the vector but also the lack of physiological regulation of the expressed insulin. Advances in understanding the developmental biology of ß-cells and the transcriptional cascade that drives it have enabled both in vivo and *ex vivo* gene therapy combined with cell therapy to be used in animal models of diabetes with success. The associated developments in the stem cell biology, immunology and gene transfer vectors have opened up further opportunities for gene therapy to be applied to target type 1 diabetes.

4.1. Cell therapy for type 1 diabetes

The considerable manipulations that are required to convert non-ß-cells into efficient glucose-sensing, insulin-secreting cells have led investigators into considering means of expanding adult or neonatal ß-cells or of harnessing the developmental potential of islet precursors cells and of embryonal stem cells.

4.2. Stem cells

A stem cell is, by definition, the one cell capable of duplicating itself and resuming its undifferentiated status, while also originating progeny that can differentiate into one or more final products that are physiologically defined by their specific functions (Wagers and Weissman, 2004). Proceeding through the differentiation pathway, stem cells can be categorized as totipotent, pluripotent, multipotent, oligopotent, and unipotent, depending upon all their possibly reversible, progressively

acquired characteristics (Fig. 2) (Wagers and Weissman, 2004).

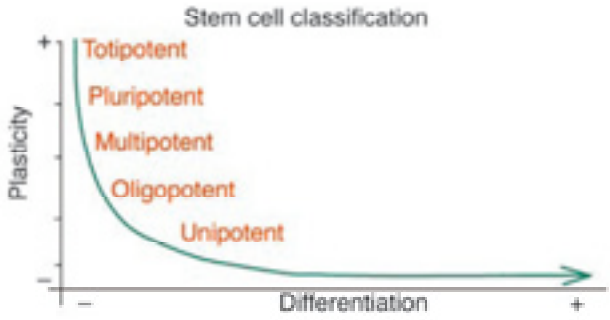


Figure 2. Classification of stem cells based on their developmental potential according to Wagers and Weissman (Wagers and Weissman, 2004). Totipotent, able to give rise to all embryonic and extraembryonic cell types; pluripotent, able to give rise to all cell types of the embryo proper; multipotent, able to give rise to a subset of cell lineages; oligopotent, able to give rise to a restricted subset of cell lineages; unipotent, able to contribute only one mature cell type.

4.2.1. Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent cell lines derived from the inner cell mass of blastocyst-stage embryos (Shamblott *et al.*, 1998; Thomson *et al.*, 1998; Cowan *et al.*, 2004; Hwang *et al.*, 2004) and their differentiation in culture may reproduce characteristics of early embryonic development. It has been shown that *in vitro* differentiation of mouse ESC can generate embryoid bodies, which, after selection for nestin-expression, can be stimulated to differentiate towards a ß-cell-like phenotype (Lumelsky *et al.*, 2001). Variations in ESC-culture conditions can generate cells with properties of ß-cells (Kahan *et al.*, 2003; Kania *et al.*, 2003; Stoffel *et al.*, 2004) and the expression of transcription factors, such as pax4 or pdx-1, have yield promising results differentiating ESCs into ß-like cells (Blyszczuk *et al.*, 2003; Miyazaki *et al.*, 2004). In these experiments, the early and uncontrolled introduction of transcription factors into ESC during *in vitro* differentiation did not yield encouraging results (Stoffel *et al.*, 2004), whereas regulated expression of the introduced transcription factors might be more successful (Miyazaki *et al.*, 2004). However, some doubt has been cast on whether ESC differentiation protocols truly yield cells that produce insulin, or cells that

merely absorb insulin from the medium (Rajagopal *et al.*, 2003). On the other hand, it has been demonstrated that differentiated cells do synthesize and release insulin, leading to reversal of diabetes in rodents after transplantation of ESC derived insulin-producing cells (Hori *et al.*, 2002; Blyszczuk *et al.*, 2003). Similarly, ESC genetically selected for insulin expression and injected into diabetic rats improved glucose control (Soria *et al.*, 2001). However, to induce differentiation of human ESC in order to produce insulin particular culture conditions are needed (Assady *et al.*, 2001; Segev *et al.*, 2004). In this regard, human ESC cell culture techniques that do not require murine feeder cells have been developed, allowing for single species propagation of ESC and avoiding possible zoonotic infection of cells intended for clinical use (Moritoh *et al.*, 2003). With the prospect of *in vivo* ESC transplantation, possible hazards and problems such as control of differentiation and teratoma formation, still remain to be overcome (Sipione *et al.*, 2004). Nevertheless, existing ESC lines are far from being ideal for generating ß-cells and additional ESC lines continue to be generated (Cowan *et al.*, 2004). Finally, in addition to scientific hurdles, ethical concerns about the use of ESC need to be addressed and resolved in the face of this powerful technology.

4.2.2. Adult stem cells

4.2.2.1. Adult stem cells isolated from the pancreas, liver or intestine.

Progenitor cells resident in isolated pancreatic tissue might be able to give rise to endocrine islet cells. Human and rodent pancreatic-duct cells (Bonner-Weir *et al.*, 2000; Ramiya *et al.*, 2000) islet-derived cells (Teitelman, 1996; Zulewski *et al.*, 2001) and exocrine tissue (Lipsett and Finegood, 2002) contain cells that can differentiate towards a pancreatic endocrine phenotype. These tissues, cultured and differentiated *in vitro*, have been transplanted and can reverse diabetes mellitus in rodents. Interestingly, it has

been recently shown that non-endocrine epithelial cells obtained from the human pancreatic tissue discarded after islet isolation, can differentiate into insulin producing cells (Hao *et al.*, 2006). However, to induce this differentiation co-transplantation with fetal human islet-like cell clusters under the kidney capsule of mice recipients was required. Therefore, a bone-fide pancreatic stem cell for ß-cell regeneration remains elusive. A genetic-marking study in mice casts doubt on the existence of any ß-cell progenitor cells and suggests that ß-cells regenerate only by proliferation of existing ß-cells (Dor *et al.*, 2004). In human beings, early immunological intervention to stop ß-cell destruction during the development of type 1 diabetes mellitus allows recovery of pancreatic endocrine function (Herold *et al.*, 2002; Glandt *et al.*, 2003). This finding might in part be attributable to recovery in ß-cell mass by recruitment of local pancreatic or extrapancreatic progenitor cells that differentiate into cells and/or proliferation of remaining ß-cells during protection from immune-mediated destruction. Similarly, in NOD mice treated with Freud's complete adjuvant, the immune response against ß-cells was blocked and this intervention allowed recovery from diabetes by increasing ß-cell mass (Kodama *et al.*, 2003; Chong *et al.*, 2006; Nishio *et al.*, 2006; Suri *et al.*, 2006).

4.2.2.2. Bone marrow stem cells

Bone marrow harbours cells that can become parenchymal cells after entering the liver, intestine, skin, lung, skeletal muscle, heart muscle, and central nervous system (Herzog *et al.*, 2003) in rodent models and in human recipients of marrow or organ transplantation (Theise *et al.*, 2000; Korbling *et al.*, 2002). In rodents, haemopoietic organs harbour cells that can also differentiate into functional pancreatic endocrine cells (Ryu *et al.*, 2001; Hess *et al.*, 2003; Ianus *et al.*, 2003; Kodama *et al.*, 2003; Zorina *et*

al., 2003; Kofman *et al.*, 2004). For example, in one study, donor-derived cells were found in pancreatic islets of recipient mice 1-2 months after bone-marrow transplantation, (Ianus *et al.*, 2003). These cells expressed insulin and genetic markers of ß-cells. When cultured, the donor-derived ß-like cells were shown to secrete insulin in response to glucose, and demonstrated intracellular calcium fluctuations similar to normal ß-cells. The authors demonstrated that 1–3% of the islet cells originated from the transplanted marrow in the studied period (Ianus *et al.*, 2003). Since a marrow-derived cell-type with pluripotential capacity to transdifferentiate into various phenotypes has been described (Jiang *et al.*, 2002a), this or a similar cell type might be able to differentiate into pancreatic ß-cells. Similar transplantation experiments have been done in overtly diabetic mice whose ß-cells have been destroyed by streptozotocin (STZ)(Hess *et al.*, 2003). After bone-marrow transplantation, blood glucose and insulin concentrations were normal, and survival of animals was better (Hess *et al.*, 2003). In islets, marrow-derived cells were shown to have differentiated into endothelial cells and, more rarely, into insulin-expressing cells (Hess *et al.*, 2003). In these experiments, endothelial engraftment was speculated to stimulate the proliferation of local pancreatic progenitors, leading in turn to the increased insulin-producing cell mass. Although these studies show the possibility of bone marrow transplantation as a therapeutic approach for ß-cell replacement, the immunological destruction of newly regenerated ß-cells in type 1 diabetes remains a problem.

Bone-marrow transplantation can be used to induce microchimerism. Thus, in non-obese diabetic (NOD) mice, an autoimmune model of type 1 diabetes, transplanted with marrow before development of autoimmune diabetes, chimerism prevented

development of diabetes mellitus, presumably by mechanisms involving donor immunoregulatory cells preventing the host cells from becoming autoreactive against ß-cells. By contrast, in NOD mice that were already diabetic, induction of chimerism by sublethal or lethal irradiation followed by marrow transplantation did not result in recovery from diabetes (Zorina *et al.*, 2003). However, when these diabetic transplant-recipients were kept normoglycaemic by insulin therapy, they ultimately recovered from diabetes. Analysis of pancreatic tissue showed increased proliferative activity and regeneration of ß-cells. Thus, marrow transplantation to induce immunological control plus maintenance of normoglycaemia allowed local or progenitor cells to proliferate as an adaptive response (Zorina *et al.*, 2003).

Transplantation of mesenchymal cells from the spleen combined with Freund's complete adjuvant led to reversal of diabetes accompanied by regeneration of insulin-producing islets (Ryu *et al.*, 2001) The transplanted splenic mesenchymal cells differentiated into ß-cells (Kodama *et al.*, 2003). Thus splenic mesenchymal cells transplanted under certain conditions seem not only to keep immune destruction of islets in check, but also could transdifferentiate into pancreatic ß-cells. However, recent experiments demonstrated that restoration of b cell mass using this protocol was not due to transdifferentiation of splenocytes into b cells as the cause for b cell mass restoration (Chong *et al.*, 2006; Nishio *et al.*, 2006; Suri *et al.*, 2006).

Bone marrow cells can differentiate *in vitro* under controlled conditions into insulin expressing cells (Jiang *et al.*, 2002a; Jahr and Bretzel, 2003) and such cells, transplanted under the kidney capsule of diabetic rodents, correct hyperglycemia. Removal of the grafted kidney returned the animals to a diabetic state (Oh *et al.*, 2004) Cell fusion has been suggested as a mechanism of apparent adaption of bone-marrow-derived cells into an extramedullary phenotype (Wagers *et al.*, 2002; Kofman *et al.*,

2004) Studies involving pancreatic endocrine-cell differentiation from haemopoietic-organ derivatives largely rule out cell-fusion events as a mechanism of transdifferentiation (Ryu *et al.*, 2001; Hess *et al.*, 2003; Ianus *et al.*, 2003; Kodama *et al.*, 2003; Zorina *et al.*, 2003; Hao *et al.*, 2006). Bone marrow-derived extramedullary parenchymal tissue is not always found (Theise and Wilmut, 2003). Importantly, several groups found little if any transdifferentiation of bone marrow into ß-cells (Choi *et al.*, 2003; Lechner *et al.*, 2004; Taneera *et al.*, 2006) or bone marrow derivation of intra-islet endothelial cells in diabetic mice (Lechner *et al.*, 2004). One group reported the generation of insulin-producing cells in liver, adipose tissue, spleen, and bone marrow in rodent models of diabetes mellitus (Kojima *et al.*, 2004). Bone marrow transplantation showed that most if not all extrapancreatic insulin-producing cells derive from donor bone-marrow (Kojima *et al.*, 2004).

5. GENE THERAPY.

5.1. Introduction to gene therapy.

The major goal of gene therapy is to introduce a functional gene in a target cell and restore protein production that is absent or deficient due to a genetic disorder. A key factor in the success of gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues, without causing any pathogenic effect. Over the years, a number of gene transfer vehicles have been developed that can roughly divided into two categories: non-viral and viral vectors. Non-viral vector gene delivery systems include direct injection of naked DNA and encapsulation of DNA with cationic lipids (liposomes) as delivery systems to introduce the genetic material into a target cell. Viral delivery systems exploit the capacity of viruses to enter the target cells, transfer the DNA to the cell nucleus and express proteins.

5.2. Viral vectors.

Considered by some to be among the simpler forms of life, viruses represent highly evolved natural vectors for the transfer of foreign genetic information into cells. This attribute has led to extensive attempts to engineer recombinant viral vectors for the delivery of therapeutic genes into diseased tissues.

The viral life cycle can be divided into two temporally distinct phases: infection and replication. Infection results in the introduction of the viral genome into the cell. This leads to an early phase of gene expression characterized by the appearance of viral regulatory products, followed by a late phase, when structural genes are expressed and assembly of new viral particles occurs. In the case of gene therapy vectors, the viral

particles encapsulate a modified genome carrying a therapeutic gene cassette in place of the viral genome. Transduction is defined as the abortive (non-replicative or dead-end) infection that introduces this functional genetic information expressed from the recombinant vectors into the target cell (Fig. 3).

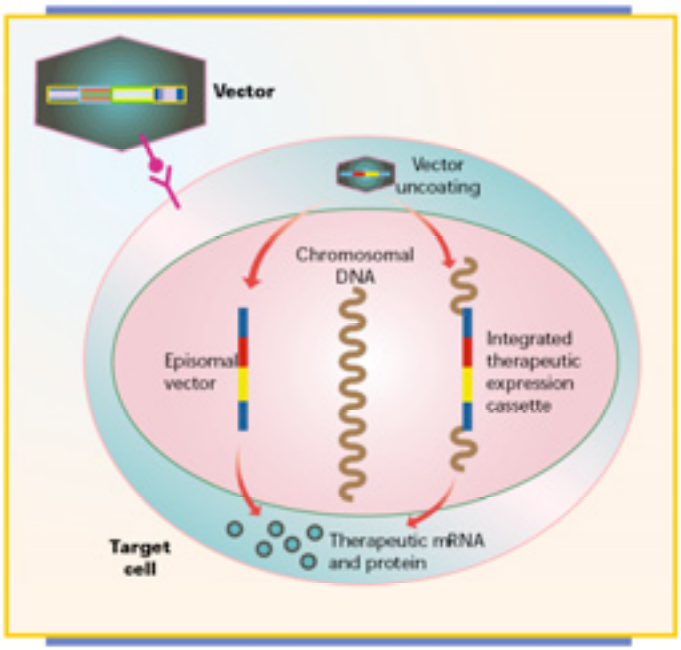


Figure 3. Transduction of the target cell. The vector particle containing the therapeutic gene sequences binds to a cell, generally through a receptor mediated process and then enters the cell, allowing the genome to enter the nucleus. The vector genome may go through complex processes but ends up as dsDNA that, depending on the vector, can persist as an episome or become integrated into the host genome. Expression of the therapeutic gene follows.

The relative concentration of vectors is measured as a titer expressed as the concentration of viral particles and/or the number of virions that are capable of transduction. The transducing particles usually represent a small percentage of total particles, and can vary between different preparations. Particle titer and an infectious or transducing titer are both important, because impurities and variations in infectious activity can influence efficacy, toxicity and immunogenicity.

For gene therapy to be successful, an appropriate amount of a therapeutic gene must be delivered into the target tissue without substantial toxicity. Each viral vector system is characterized by an inherent set of properties that affect its suitability for specific gene therapy applications. For some disorders, long-term expression from a relatively small proportion of cells would be sufficient (for example, some genetic disorders), whereas other pathologies might require high, but transient, gene expression. Problems that may be observed with gene transfer vectors include acute toxicity from the infusion of foreign materials, cellular immune responses directed against the transduced cells, humoral immune responses against the therapeutic gene product and the potential for insertional mutagenesis by certain integrating vectors.

Viral vectors currently available for gene therapy are based on different viruses and can roughly be categorized into integrating and nonintegrating vectors. Vectors based on retroviruses, including lentivirus and foamy virus, have the ability to integrate their viral genome into the chromosomal DNA of the host cell, which will possibly achieve life-long gene expression. Wild type adeno-associated virus are integrating vectors, however, recombinant AAV vectors have lost this property because of the lack of viral proteins in the recombinant genome. Then, AAV can be essentially considered as a non-integrating vector although few cases of non-specific integration have been described (Nakai *et al.*, 2003). Vectors based on adenovirus and herpes simplex virus type 1 (HSV-1) represent non-integrationg vectors. The non-integrating vectors deliver their genomes into the nucleus of the target cell, where they remain episomal.

5.2.1. Adenoviral vectors.

5.2.1.1. Adenovirus structure and biology.

Adenovirus was first isolated from human adenoid cells more than 50 years ago (Rowe *et al.*, 1953). Since then, more than 100 different adenovirus species have been identified from various mammals, birds, and reptiles and all are characterized by a distinctive architecture and common chemical composition (Büchen-Osmond, 2003).

Human adenoviruses are classified into six subgroups (A–F), which are further divided into serotypes based on their immunological properties. Serotype 2 (Ad2) and serotype 5 (Ad5) of subgroup C, which are the most extensively studied of the human adenoviruses, are found worldwide. The adenovirus virion is a nonenveloped icosahedral particle about 70–90 nm in size with an outer protein shell surrounding an inner nucleoprotein core (Fig. 4).

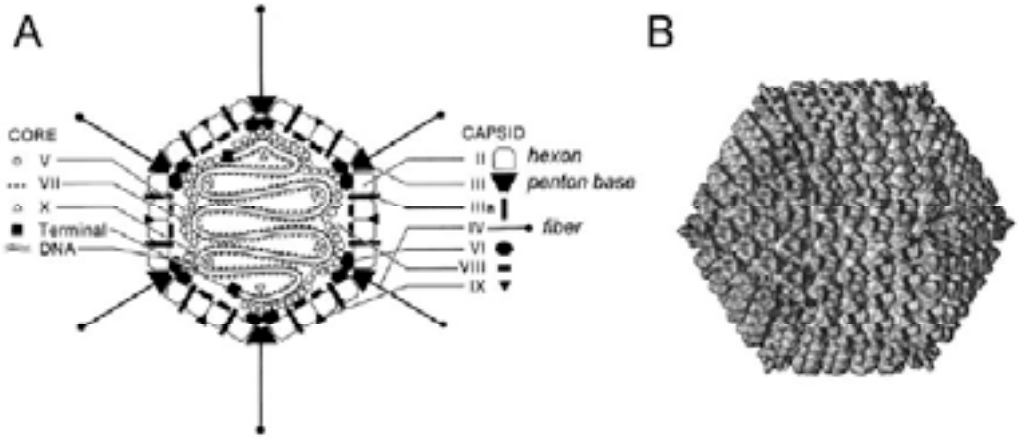


Figure 4. (**A**) Section of the adenovirus virion, indicating the relative orientations of the protein components and the viral DNA. The core and capsid proteins are listed separately (Stewart *et al.*, 1993). (**B**) A 15-Å resolution three-dimensional cryo-EM image reconstruction of Ad5 viewed along the 3-fold axis of the icosahedral capsid The trimeric towers of hexon, the major coat protein, create the features visible on the facets. The fiber protrudes from the penton base at each 5-fold vertex. Only a short portion of the long fiber is visible; the rest is washed out when averaged by the reconstruction process.

The facets of the virus capsid are composed primarily of trimers of the hexon protein, as well as a number of other minor components including protein IIIa (pIIIa), pVI, pVIII, and pIX. The capsid vertices consist of the penton base, which acts to anchor the fiber protein, the moiety responsible for primary attachment of virions to the cell surface.

Adenovirus cores contain the viral DNA as well as pV, mu, and the histone-like protein pVII. The genome itself is a linear, double-stranded DNA that is approximately 36 kb long. Each end of the genome has an inverted terminal repeat (ITR) of 100–140 bp to which the terminal protein is covalently linked. Genes are encoded on both strands of the DNA in a series of overlapping transcription units (Fig. 5).

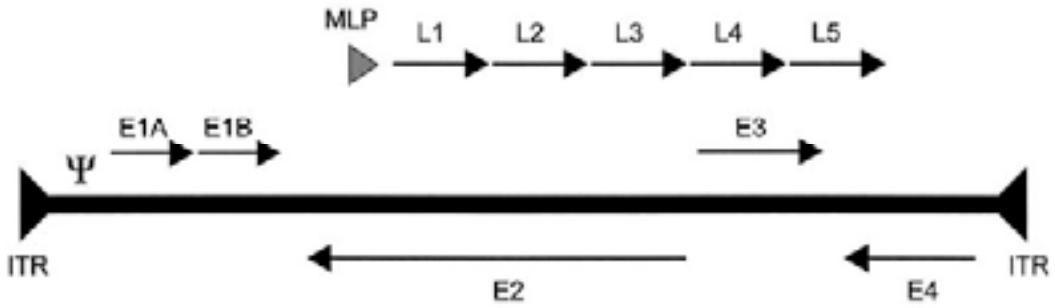


Figure 5. Map of the adenovirus genome and transcription units. The central, solid line represents the viral genome. Positions of the left and right ITRs, the packaging sequence (Ψ), the early transcription units (E1A, E1B, E2, E3, and E4), and the major late transcription unit (major late promoter [MLP], L1–L5) are shown. Arrows indicate the direction of transcription.

For all groups, except group B adenoviruses, initial attachment of virion particles to the cell surface occurs through binding of the fiber knob to the coxsackievirus B and adenovirus receptor (CAR) (Bergelson *et al.*, 1997). After initial attachment to the cell surface, an exposed RGD protein motif on the penton base interacts with members of the v integrin family, triggering virus internalization by clathrin-dependent, receptor-mediated endocytosis (Stewart *et al.*, 1997; Meier *et al.*,

2002).

The first viral transcription unit to be expressed is E1A. As with almost all adenovirus transcription units, E1A produces multiple mRNA and protein products by way of differential mRNA processing. During infection, the E1A proteins function to *trans*-activate the other adenovirus early transcription units (E1B, E2, E3, and E4) and to induce the cell to enter S phase in order to create an environment optimal for virus replication (Berk, 1986). The E2 region encodes proteins necessary for replication of the viral genome: DNA polymerase, preterminal protein, and the 72-kDa single-stranded DNA-binding protein (de Jong *et al.*, 2003). Products of the viral E3 region function to subvert the host immune response and allow persistence of infected cells (Gooding *et al.*, 1991; Bruder *et al.*, 1997; Bennett *et al.*, 1999). The E4 transcription unit encodes a number of proteins that have been known to play a role in cell cycle control and transformation (Tauber and Dobner, 2001). Most adenovirus late genes are expressed from five regions, L1–L5, and are transcribed from one promoter, the major late promoter (MLP). These transcripts primarily encode structural proteins of the virus and other proteins involved in virion assembly. The packaging sequence itself is a series of seven repeats at the left end of the genome (Hearing *et al.*, 1987) which mediates genome encapsidation (Grable and Hearing, 1990). Cell lysis and release of progeny virions occur approximately 30 hr postinfection in a process involving the E3-11.6K protein, also called the adenovirus death protein (ADP) (Tollefson *et al.*, 1996).

Several types of adenoviral vectors have been developed and extensively used in different gene therapy approaches.

5.2.1.2 First and second generation adenoviral vectors.

The initial strategy used in the construction of adenovirus vectors was the replacement of the E1 region with the desired transgene. Since E1 region are necessary for activation of viral promoters and expression of both early and late genes, removal of the E1 coding sequence results in viruses that are severely impaired in their ability to replicate. This strategy give raise the so-called first generation (FG) adenoviral vectors. The ability to delete the E1 region is made possible by the existence of cell lines that provide these functions *in trans*. The classic cell line for this purpose is the 293 cell line, a human embryonic kidney-derived line that has been transformed by the adenovirus E1 region (Graham *et al.*, 1977). The production of FG vectors was initially carried out by homologous recombination in mammalian cells between constructs carrying the left and right ends of the genome (Chinnadurai *et al.*, 1979). However, this method proved to be inefficient and has prompted the development of techniques relying on standard cloning in bacteria and subsequent transfection of recombinant chromosomes into mammalian cells for virus production (Danthinne and Imperiale, 2000). Removal of the E1 region alone gives approximately 5.1 kb of cloning space without affecting viral titer and growth rate (Bett *et al.*, 1993). In addition to E1 deletion, many of the first-generation vectors also contain a deletion in the E3 region, which is not essential for *in vitro* production of the vectors, in order to increase cloning capacity up to 8.2 kb.

The main problem associated with the use of FG vectors is their stimulation of a cellular immune response, resulting in the destruction of transduced cells that are expressing therapeutic transgenes. Indeed, a number of early studies showed that administration of E1-deleted vectors to immune-competent animals results in only transient transgene expression (Yang *et al.*, 1994a; Dai *et al.*, 1995; Yang *et al.*, 1995). It is theorized that the immune response is stimulated by low levels of replication that

can occur even in the absence of the E1 genes. This idea is supported by findings that genome replication and late gene expression can occur from E1-deleted vectors *in vivo* (Yang *et al.*, 1994a; Yang *et al.*, 1994b). Therefore, the use of first-generation vectors is precluded for applications requiring short term gene expression such as cancer therapy and vaccination.

To prevent the immune response generated by FG adenoviral vectors, a second generation was constructed by the removal of E2 and E4 coding sequences. These vectors provide larger capacity for transgene insertion and reduced immunity. However, the major drawback encountered during construction of these multiply deleted viruses is the need for isolation of cell lines expressing the missing functions *in trans* (Schaack *et al.*, 1995; Amalfitano *et al.*, 1996; Gorziglia *et al.*, 1996; Amalfitano and Chamberlain, 1997) Nevertheless, the advantages of second generation Ad over FGAd remain controversial as some studies show them to be superior in terms of toxicity and longevity of transgene expression (Engelhardt *et al.*, 1994a; Engelhardt *et al.*, 1994b; Yang *et al.*, 1994b; Goldman *et al.*, 1995; Gao *et al.*, 1996; Dedieu *et al.*, 1997; Wang *et al.*, 1997), whereas other studies do not (Fang *et al.*, 1996; Morral *et al.*, 1997; Lusky *et al.*, 1998; O'Neal *et al.*, 1998; Reddy *et al.*, 2002).

5.2.1.3. Helper dependent adenoviral vectors.

Significant improvement in the safety and efficacy of Ad-based vectors came with the development of helper-dependent adenoviral vectors (HDAds, also referred to as gutless, gutted, fully deleted, or high-capacity) which are deleted of all viral coding sequences. Comparison of first and second generation vectors with Helper-dependent vectors genomes is shown in Fig. 6. HDAds retain the advantages of FGAds, including high-efficiency *in vivo* transduction and high-level transgene expression. However,

owing to the absence of viral gene expression in transduced cells, these HDAds are able to mediate high-level, long-term transgene expression in the absence of chronic toxicity (Morral *et al.*, 1998; Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Morral *et al.*, 1999; Balague *et al.*, 2000; Cregan *et al.*, 2000; Kim *et al.*, 2001; Maione *et al.*, 2001; Oka *et al.*, 2001; Zou *et al.*, 2001; Ehrhardt and Kay, 2002; Reddy *et al.*, 2002) (Gilbert *et al.*, 2002; Gilbert *et al.*, 2003; Dudley *et al.*, 2004; Fleury *et al.*, 2004; Pastore *et al.*, 2004; Wen *et al.*, 2004; Brunetti-Pierri *et al.*, 2005; Mian *et al.*, 2005; Toietta *et al.*, 2005). Other advantages of these vectors include lack of germ line transmission and insertional mutagenesis because the vector genome exists episomally in transduced cells. Moreover, the deletion of the viral sequences permits a large cloning capacity of 37 kb, allowing for the delivery of whole genomic loci, multiple transgenes, and large *cis* acting elements to enhance, prolong, and regulate transgene expression.

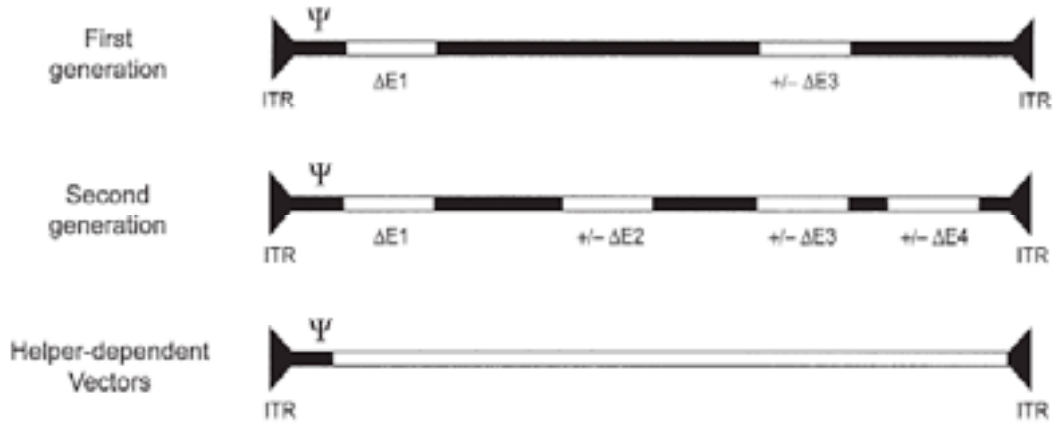


Figure 6. Genome structure of first-generation, second-generation, and helper-dependent vectors. Regions that have been deleted are indicated by open boxes.

Because HDAds are deleted of all viral coding sequences, a helper virus is required for their propagation. The first efficient method for generating HDAds was the Cre–*loxP* system developed by Parks and coworkers in 1996 (Parks *et al.*, 1996) (Fig.

7). In this system the HDAd genome is first constructed in a bacterial plasmid. Minimally, the HDAd genome contains the expression cassette of interest and 500 bp of *cis*-acting Ad sequences necessary for vector DNA replication (inverted terminal repeats, ITRs) and packaging (Ψ). Because efficient packaging into the Ad capsid requires a genome size between 27.7kb (Parks and Graham, 1997) and 38kb (Bett *et al.*, 1993), "stuffer" DNA is usually included in HDAds. To generate the HDAd viral particles, the plasmid is first digested with the appropriate restriction enzyme to liberate the HDAd genome from the bacterial plasmid sequences. 293 cells expressing the site-specific recombinase Cre are then transfected with the linearized HDAd genome and subsequently infected with the helper virus. The helper virus, bears a packaging signal flanked by *loxP* sites, the target sequence for Cre, and thus after infection of 293Cre cells the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the *loxP* sites. This renders the helper viral genome unpackageable, but still able to undergo DNA replication and thus *trans*-complement the replication and encapsidation of the HDAd genome. The titer of the HDAd is amplified by serial coinfection of 293Cre cells with the HDAd and the helper virus, and the HDAd is finally purified by CsCl ultracentrifugation.

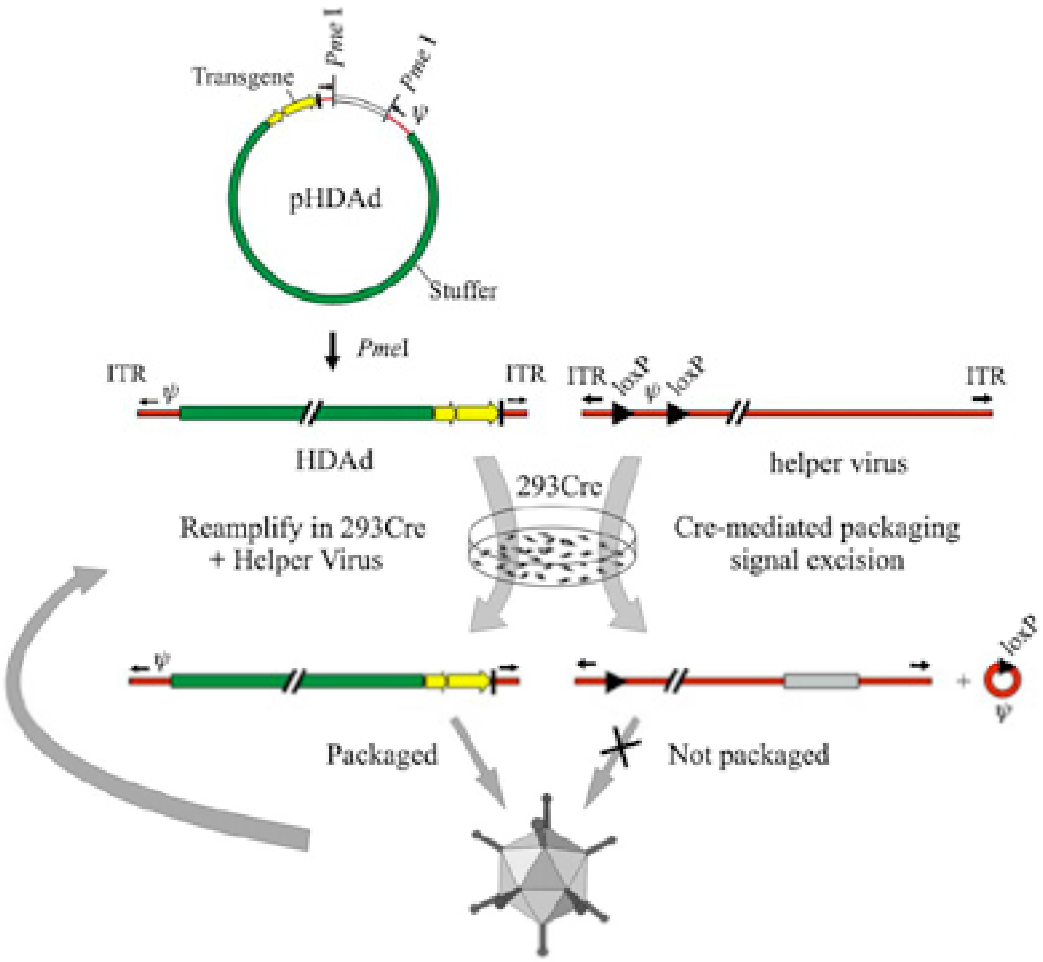


Figure 7. The Cre–*loxP* system for producing HDAds. The HDAd contains only 500 bp of *cis*-acting Ad sequences required for DNA replication (ITRs) and packaging ( ); the remainder of the genome consists of the desired transgene and non-Ad stuffer sequences. The HDAd genome is constructed as a bacterial plasmid (pHDAd) and is liberated by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd, the liberated genome is transfected into 293Cre cells and infected with a helper virus bearing a packaging signal (*Ψ*) flanked by *loxP* sites. Cre-mediated excision of *Ψ*) renders the helper virus genome unpackageable, but still able to replicate and provide all of the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HDAd is increased by serial coinfections (called passages) of 293Cre cells with the HDAd and the helper virus. Finally, the HDAd is purified by CsCl ultracentrifugation.(Parks *et al.*, 1996)

Major hurdless in the gutless vector generation include low efficiency in the production methods, genome instability, generation of replication competent particles and helper virus contamination. Since the original Cre-lox system was described others systems have been generated to circumvent these limitations ((Palmer and Ng, 2003; Sakhuja *et al.*, 2003; Nakano *et al.*, 2005; Oka and Chan, 2005; Shi *et al.*, 2006)). A production system composed of a suspension-adapted producer cell line that expressed

high levels of Cre, a helper virus resistant to mutation, and refined protocols led to production of titers $>10^{13}$ viral particles and with exceedingly low helper virus contamination of 0.4–0.1% without relying on CsCl purification and 0.02–0.01% after CsCl purification (Palmer and Ng, 2003).

Encouraging results have been obtained with HDAd in several animal model of disease. These vectors have been used extensively for liver-directed gene therapy because of their ability of transduce hepatocytes after systemic intravenous administration. HDAd-mediated gene therapy in baboons using vectors carrying the human α1-antitrypsin (hAAT) gene, showed expression of hAAT for more than 1 year in two of the three animals (Morral *et al.*, 1999). Many other studies have exploited the advantages of HDAd for hepatic transduction (Ehrhardt and Kay, 2002; Belalcazar *et al.*, 2003; Kojima *et al.*, 2003; Mian *et al.*, 2004; Wang *et al.*, 2004b; Toietta *et al.*, 2005). In addition to the liver, gene transfer to other tissues such as the brain (Zou *et al.*, 2001), eye (Kreppel *et al.*, 2002b; Oshima *et al.*, 2004), muscle (Chen *et al.*, 1997; Chen *et al.*, 1999; Jiang *et al.*, 2001; Maione *et al.*, 2001; Gilbert *et al.*, 2002; Gilchrist *et al.*, 2002; Jiang *et al.*, 2002b; Gilbert *et al.*, 2003; Fleury *et al.*, 2004; Jiang *et al.*, 2004), and lung (Toietta *et al.*, 2003; Koehler *et al.*, 2006) has been described using these vectors.

5.2.2. Adeno-associated viral vectors.

Adeno-asociated virus (AAV) was originally identified as a contaminant of adenovirus cultures. AAV is a member of the dependovirus genus of the family Parvoviridae. Like other parvoviruses, AAV exists as a non-enveloped icosahedral particle with a diameter of approximately 20 nm. AAV has never been shown to cause any human disease, despite a high seroprevalence rate. Multiple serotypes of AAV have

been identified, including human serotypes 1,2,3 and 5, simian serotype 4, as well as bovine, canine and avian AAV (Blacklow, 1988). However, since each serotype could have different cell tropism, many efforts are focused in the isolation and characterization of new serotypes to look for a tissue-specific serotype to be used as gene transfer vector. Thus, AAV serotype 6 was chracterized in 1999 and appears to be a recombinant between AAV1 and AAV2 (Xiao *et al.*, 1999. Later, four new serotypes were isolated from nonhuman primates AAV7, AAV8, AAV9 and rh10 [Gao, 2002 #684; Gao *et al.*, 2002).

The AAV2 genome has been cloned (Samulski *et al.*, 1982; Laughlin *et al.*, 1983), sequenced (Srivastava *et al.*, 1983), and characterized in detail, and this is the most widely use for the generation of recombinant vectors. The termini consist of 145-nucleotide long ITRs. The ITRs contain all cis-acting functions required for DNA replication, packaging integration, and subsequent excision and rescue (Samulski *et al.*, 1989; Srivastava *et al.*, 1989). Internal to the ITR are two viral genes; *rep*, which encodes functions required for replication, and *cap*, which encodes structural proteins of the capsid. A total of four Rep proteins (Rep78, Rep68, Rep52, and Rep40) and three cap proteins (VP1, VP2, and VP3) are generated by alternative splicing. The AAV have the tendency to integrate within a specific region of human chromosome 19, the AAV-S1 site (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Kotin *et al.*, 1992; Samulski, 1993). The mechanism for integration may involve the Rep68 protein (Weitzman *et al.*, 1994).

Construction of AAV recombinant vectors can be done by several methods; however, five elements are generally required: (a) cells permissive for AAV replication (e.g. 293 cells), (b) a helper virus (e.g. adenovirus, or a plasmid including the adenovirus sequences), (c) a recombinant AAV vector of 5 kb or less, including ITRs

flanking the transgene and any promoter, enhancer, or polyadenylation elements, (d) a source of rep proteins, and (e) a source of capsid proteins. These elements can be supplied by co-transfection two or three plasmids. Cells must be then be lysed by serial freeze-thaw or other physical methods to release the packaged virions. Packaged AAV vector can then be separated from cellular debris by CsCl gradient ultracentrifugation.

The principal advantage of AAV over other DNA virus, such as adenovirus, is the lack of any viral coding sequence within the vector, which prevents transduced cells from being recognized and rejected by the immune systems. In addition, AAV is also efficient at cell entry and tends to persist in cells over long periods of time. Although the low immune reponse was true in animal models, it has been shown that AAV vectors could trigger an immune response in humans (Manno *et al.*, 2006).

6. GENE THERAPY FOR TYPE 1 DIABETES.

Limitions of the current therapy of insulin injections and islet transplantation can be potentially circumvented with gene therapy. Indeed, advances in cell and gene therapy have rekindled the possibility of not only ameliorating, but also potentially curing diabetes. Gene therapy strategies for diabetes can broadly be divided into three categories: preventive, adjunctive and curative. While preventive and adjunctive strategies primarily address the autoimmune pathogenesis of type 1 diabetes and the immune response to islet grafts post-transplantation, respectively, curative strategies involve restoring insulin production and secretion by effecting insulin transgene expression or by inducing islet neogenesis.

6.1. Gene therapies aimed at reducing immune reactions or inducing immune tolerance.

Primary prevention of type 1 diabetes involves identifying patients at risk and instituting measures to prevent the autoimmune destruction of ß-cells such as immunomodulatory gene therapy. This has been shown to work in rodent models (Jun *et al.*, 2002; Flodstrom-Tullberg *et al.*, 2003; Goudy *et al.*, 2003; Steptoe *et al.*, 2003; Jin *et al.*, 2004; Song *et al.*, 2004). These strategies were aimed at inducing tolerance or hyporesponsiveness to islet autoantigens by either increasing tolerance-inducing regulatory T lymphocytes (IL-10 gene therapy (Goudy *et al.*, 2003); by suppressing reactive T lymphocytes (immune therapy using recombinant vaccinia virus expressing glutamic acid decarboxylase (Jun *et al.*, 2002); by targeting T lymphocytes for apoptosis (Jin *et al.*, 2004); by inducing tolerance in ß-cells (by overexpression of suppressor of cytokine signaling-1 (SOCS-1) (Flodstrom-Tullberg *et al.*, 2003); or by inducing antigen-presenting cells to express proinsulin, an autoantigen (Steptoe *et al.*, 2003). However, the translation of the animal experiments to human trials is constrained by the lack of markers with acceptable positive and negative predictive values to unequivocally identify and select appropiate patients who are at risk for preventive therapy. Yet, conceptually, this is the area that will need to be explored if our ultimate goal is the primary prevention of type 1 diabetes. Preventive strategies have currently focused on secondary prevention, that is, measures to prevent the further loss of ß-cells once diabetes has set in. These are instituted optimally at diagnosis or in the honeymoon period following the initial acute presentation as often seen in type I diabetes (Herold *et al.*, 2002; Kodama *et al.*, 2003; Keymeulen *et al.*, 2005; Chong *et al.*, 2006; Nishio *et al.*, 2006; Suri *et al.*, 2006).

6.2. Genetic manipulation of extra-pancreatic cells to produce insulin.

There have been substantial efforts to engineer glucose-responsive insulin secretion into cells other than ß-cells. For instance, gut K cells of the mouse were induced to produce human insulin by transfecting them with the human insulin gene linked to the 5'-regulatory region of the gene encoding glucose-dependent insulinotropic polypeptide (GIP) (Cheung *et al.*, 2000). Rodent-liver stem cells and human fetal-liver cells have also been differentiated *in vitro* into insulin-secreting cells by introduction of ß-cell-specific genes. These experiments demonstrating differentiation to ß-like cells from adenovirus-transduced murine hepatocytes with the *Pdx1* gene, alone (Ferber *et al.*, 2000) or in combination with *NeuroD* (Kojima *et al.*, 2003), attracted significant attention and has inspired new hope. Importantly, tranduction of human hepatocytes with *pdx1* gave similar results and insulin production was also observed (Zalzman *et al.*, 2003; Sapir *et al.*, 2005; Zalzman *et al.*, 2005). Sufficient levels of insulin were secreted to satisfy the needs of a diabetic mouse, which became, and remained, steadily euglycemic after treatment with *pdx1* transduced cells.

6.3. Gene transfer to the pancreas and/or ß-cells and its applications.

Efficient and stable gene transfer to pancreatic cells is required for pursuing gene therapy strategies for ß-cell expansion and modification. To genetically engineer the endocrine pancreas the selection of both the vector and the route of administration are key. While several vectors can be used to transfer foreign genes to pancreatic islets and to pancreatic ß-cell lines *in vitro,* few attempts have been assayed to target the islets *in vivo*.

The shortage of islets donors, together with the need of more than one donor per each recipient have increase the interest in genetic manipulations of the islets to avoid

the immune response and improve ß-cell function upon transplantation. An appreciable amount of work was focused on using viral vectors to infect intact islets in culture prior to transplantation into recipients to impede the allogenic rejection.

6.3.1. Gene transfer to pancreatic islets in vitro.

A large number of vectors have been used to date to transduce intact islets *in vitro*, a list of these attempts is included in Table 1. However, no ideal vector exists and the pros and cons are listed in Table 2. In addition to the introduction of key genes to the islets by gene transfer, new technologies based on small interference RNA (siRNA) will also allow downregulation of undesired genes in ß-cells (Bain *et al.*, 2004; Schisler *et al.*, 2005). In addition, new generation vectors based on AAV-ITR plasmids, novel classes of lentivirus, lentivirus-herpesvirus hybrids, as well as helper-dependent adenoviral vectors are promising tools, however, the efficiency and the degree to which these vectors can contribute to post-transplantation inflammation is still unknown.

Table 1. Gene transfer vectors which transduce islets in vitro (with references)

Plasmid DNA (Welsh *et al.*, 1990; Benhamou *et al.*, 1997; Gainer *et al.*, 1997; Gainer *et al.*, 1998)
Adenovirus (Becker *et al.*, 1994; Csete *et al.*, 1995; Benhamou *et al.*, 1996; Saldeen *et al.*, 1996; Muruve *et al.*, 1997; Smith *et al.*, 1997; von Herrath *et al.*, 1997; Weber *et al.*, 1997; Judge *et al.*, 1998; Yasuda *et al.*, 1998; Giannoukakis *et al.*, 1999b; Grey *et al.*, 1999; Guo *et al.*, 1999; Uchikoshi *et al.*, 1999; Giannoukakis *et al.*, 2000a; Giannoukakis *et al.*, 2000b; Moriscot *et al.*, 2000; Contreras *et al.*, 2001a; Contreras *et al.*, 2001b; Alexander *et al.*, 2002; Bertera *et al.*, 2003)
Adeno-associated virus (Yang and Kotin, 2000; Flotte *et al.*, 2001; Rehman *et al.*, 2005; Wang *et al.*, 2006)
MoLV retrovirus (Leibowitz *et al.*, 1999)
Lentivirus (Gallichan *et al.*, 1998; Ju *et al.*, 1998; Giannoukakis *et al.*, 1999a; Kobinger *et al.*, 2004)
Herpes simplex virus (Liu *et al.*, 1996; Rabinovitch *et al.*, 1999)
Cationic liposomes (Welsh *et al.*, 1990; Saldeen *et al.*, 1996; Benhamou *et al.*, 1997)
Peptide fusion domains (Mi *et al.*, 2000; Embury *et al.*, 2001; Rehman *et al.*, 2003)

Gene transfer to the whole pancreas *ex vivo* using viral vectors has also been proposed. This approach consists in intra-arterial delivery of the viral vector to the previously isolated pancreas and high transduction efficiency of the islets is reported using this technique. To date, adenoviral and AAV vectors have been assayed using this approach (Liu *et al.*, 2001).

Table 2. General characteristics of gene delivery vehicles.

Vector type	pros	cons
Plasmid DNA	Easy to engineer, grow and purify; multicistronic variants easy to engineer	Poor persistence; non specific cell targeting; poor tissue diffusion
Adenovirus	Choice vector for pilot proof-of-principle experiments; High titers easily obtained; almost all cells and tissues are transducible; cell retargeting is possible	Immunogenic in vivo; non-stable transduction
AAV	Site-specific, stable integration achievable; almost absent immunogenicity; many cell types transducible	Time for transgene expression can be in the order of days
MoLV-based retrovirus	Stably integrating vector in rapidly-dividing cells; cell-type retargeting possible; good titers obtainable	Subject to chromosomal position-effect sensitivity, as well as methylation and cytokine effects on gene expression;
Lentivirus	Non-immunogenic; stably-integrating; Choice vector for non-dividing, non-cycling cells; good titers obtainable; Data support absence of replication-competent-recombinant vector particles in stocks	Clinical safety concerns with HIV-1-based vectors
Herpes simplex type-1 virus	Large genome available for multiple large size cistrons; good persistence in many cell types; cell-type retargeting possible	Inherent toxicity
Cationic liposome	Easy to manipulate; able to deliver plasmid DNA to almost all cells and tissue; non-immunogenic; cell-type non-specific; cell-type retargeting possible	Poor control of diffusion kinetics
Peptide fusion domains	Many cell-types transducible; high-level proein/peptide import; intact proteins/peptides delivered; not subject to gene regulation; targeting of specific proteins possible; high-level peptide production easily achievable; no reported immunogenicity	Short half life; subject to proteolytic degradation; large amounts require some time to generate

6.3.2. Gene transfer to the pancreas in vivo.

The pancreas is difficult to manipulate and pancreatitis is a serious concern, which has made effective gene transfer to this organ elusive. Most of the approaches are centered in the use of viral vectors and especially adenovirus and AAV, and different routes of administration have been examined using these vectors. Nevertheless, few examples of gene therapy based on these approaches have been reported to date.

Two routes of administration have been proposed: (1) direct injection of the vectors into the pancreatic parenchyma and (2) retrograde injection of the vectors using the common bile duct.

6.3.2.1 Direct injection to the parenchyma.

This technique was first described in mice in 1997, when an adenoviralvector suspension, expressing ß-gal as a marker gene, was injected in three different sites of the pancreatic parenchyma (McClane *et al.*, 1997c). Using this method, about 70% of the pancreatic cells were transduced, however, distribution was very restricted to the sites of injection. Most of the expression was found in acinar cells, although some ductal, endothelial, and peripheral islet cells were also transduced. An additional limitation was the duration of expression, since the 70% transduction rate observed after 3 days was reduced to 10% after 7 days and had completely disappeared by 28 days (McClane *et al.*, 1997c). Subsequent experiments demonstrated that the loss of expression was due to immune destruction of transduced cells because these cells expressed the adenoviral genes. Administration of the same vector into immunodeficient mice (MHC class I or RAG-2 deficient) resulted in 20% of expression 60 days after the injection (McClane *et al.*, 1997a). Deletion of $CD4^+$ T helper cells improved expression over time (40% of pancreatic cells expressed transgene at day 28 versus 5% in controls) and allows the vector to be readministered in the pancreas, albeit, inefficiently, when compared to naïve animals. Blockade of CD40 ligand (involved in T cell-dependent activation of B cells), which preserves the $CD4^+$ T helper cell population, also improves expression over time (30% of pancreatic cells express the transgene at day 28) and allows the vector to be readministered (McClane *et al.*, 1997a). This unique gene therapy approach involving insulin gene delivery to the exocrine

pancreas of immunodeficient mice demonstrated some benefits in reducing the hyperglycemia in diabetic mice.

In addition to vector-related immunologic reaction, direct injection was also associated with histologic damage such as increased edema, inflammation, cell destruction, and vacualization (McClane *et al.*, 1997b).

In addition to adenovirus, the ability of AAV vectors to transduce the pancreas *in vivo* have also been examined. The AAV serotype 8 vectors were more efficient than AAV2 in transducing the pancreas *in vivo* after direct injection, whereas AAV5 did not result in any detectable transgene expression (Wang *et al.*, 2004a). Nevertheless, this study also showed that adenoviral vectors were more efficient than AAV8 in transducing the pancreas *in vivo*. Persistence of gene expression by using AAV vectors was longer when compared to adenoviral vectors, the latter of which elicited significant leukocyte infiltration which resulted in greater than 90% loss of expression after 4 weeks (Wang *et al.*, 2004a).

6.3.2.2. Retrograde injection through the common bile duct.

Several issues still remain regarding the direct injection approach to deliver genes to the pancreas. One concern is the size and shape of the pancreas. The pancreas is a long and relatively narrow lobed structure, hence delivery of genes to the entire pancreas would require multiple sites of injection and, in addition, may not reach the deepest regions. Multiple injections may also increase the damage to the pancreas and the chance of inducing pancreatitis.

Pancreatic duct delivery of the vectors is a very attractive alternative to direct injection. This technique can be considered an adaptation of a clinically relevant surgical technique called endoscopic retrograde cholangiopancreatography (ERCP).

Pancreatic duct injection has successfully been used to deliver genes to the pancreas through a variety of carrier systems (e.g., cationic lipids, adenovirus) (Yang *et al.*, 1993; Raper and DeMatteo, 1996; Schmid *et al.*, 1998). In one of these first reports (Raper and DeMatteo, 1996), three surgical procedures were assayed: (1) injection into the common duct of 50µl of adenovirus suspension without any clamp; (2) clamping the proximal duct near to the duodenum to avoid virus entering the intestine after or during the injection; (3) clamping both the proximal area of the duct near the duodenum and also distal part of the duct near the liver, to avoid virus entering the liver. The third approach was the most efficient technique to transduce the pancreas (Raper and DeMatteo, 1996). When adenoviral vectors were injected following this approach, transduction of ductal epithelium was observed, but also acinar cells and some endocrine cells in the periphery of the islets were transduced. However, immune response against transduced cells and inflammation of pancreatic parenchymas was observed.

Similar results were obtained when such approach was used in mice (McClane *et al.*, 1997c). Some modification of this technique has been proposed, by injecting 250µl of adenoviral solution without any clamp (Taniguchi *et al.*, 2003). In the latter study, efficent transduction of ductal and acinar cells was observed, whereas no endocrine cells were found to express the marker gene.

The effect of adenovirus-mediated gene transfer of *pdx-1* and *ngn-3* transcription factors were studied in the pancreas by retograde transduction and few neogenic ß-cells were observed when *pdx-1* was used. However, this neogenesis was not sufficient to restore normoglycemia in diabetic mice (Taniguchi *et al.*, 2003). Similar experiments have been reported in which epidermal growth factor was transferred to the pancreas by ductal delivery of adenoviral vectors (Kozawa *et al.*, 2005). In this study, low volume

and slow infusion of the vector was used (15µl injected at 1.5µl/min) to preferentially transduce the epithelial cells of the pancreatic ducts. Insulin-positive cells differentiated from duct cells and replication of ß-cells was significantly increased. ß-cell mass was also increased and the glucose tolerance of diabetic mice was improved at 12 weeks after injection (Kozawa *et al.*, 2005).

Recently, it has been shown that AAV vectors can also be used to transduce the pancreas using pancreatic duct delivery (Loiler *et al.*, 2005). In this study, three different serotypes of AAV (AAV1, AAV8, AAV2), as well as two different promoters (chicken ß-actin and human insulin), were compared for their ability to deliver and produce a secreted gene product into the bloodstream of rats and mice. Rats injected with AAV1 showed the highest level of marker gene expression. In mice, AAV8 vector delivered the highest serum concentration of the marker protein. The chicken ß-actin promoter provided the highest expression in both rodent experiments. Immunohistochemical staining indicated transduction primarily of pancreatic acinar cells with either the AAV1 vector in the rat or the AAV8 vector in the mouse (Loiler *et al.*, 2005). Therefore, AAV vectors can be designed to deliver therapeutic genes efficiently to the pancreas and achieve high levels of gene expression and may be useful in treating pancreatic disorders, including type 1 diabetes.

The ERCP procedure is used frequently in humans and it may constitute an advantage to assay gene therapy approaches in the pancreas of human patients. However, this manipulation is not innocuous and about 3-40 % of the patients develop pancreatitis after the ERCP and need medical care (Freeman *et al.*, 1996; Freeman *et al.*, 2001).

7. INSULIN-LIKE GROWTH FACTOR-I (IGF-I).

7.1. IGF-I structure and function.

The insulin-like growth factors (IGFs) participate in the growth and function of almost every organ in the body (Daughaday and Rotwein, 1989). The three peptide hormones, or growth factors, in the IGF family - insulin, IGF-I, and IGF-II - have approximately 50 percent of their amino acids in common. The IGF-I is composed of 70 amino acids and has a molecular weight of 7.6kDa, whereas IGF-II have 63 amino acids and 7.4kDa of molecular weight (Van den Brande *et al.*, 1990). Insulin is synthesized in the ß-cells of the pancreas as proinsulin, which is cleaved to form insulin and C peptide. The IGFs, which are synthesized primarily by the liver, retain the C peptide and have an extended carboxy terminus (D domain) (Daughaday and Rotwein, 1989). Insulin circulates at picomolar concentrations and has a half-life of minutes. The IGFs, on the other hand, circulate at much higher (nanomolar) concentrations and are largely bound to one of six IGF-binding proteins that modulate IGF activity (Jones and Clemmons, 1995). These binding proteins, like the IGFs, are synthesized primarily in the liver.

Insulin, IGF-I, and IGF-II bind specifically to two high-affinity membrane-associated receptors that are tyrosine kinases. Insulin activates the insulin receptor, and both IGFs activate the IGF-I receptor (ruderman 1994). A third receptor, the IGF-II-mannose-6-phosphate receptor, binds IGF-II but has no known intracellular signaling actions. Activation of either the insulin receptor or the IGF-I receptor evokes similar initial responses within the cell (LeRoith *et al.*, 1992a). However, since insulin regulates metabolic functions and the IGFs regulate growth and differentiation functions, the final pathways these hormones activate within the cell must be separate and distinct.

IGFs and their binding proteins are also produced locally by most tissues, in addition to the liver, where they act in an autocrine or paracrine manner. The IGFs are important in the function of almost every organ in the body (Daughaday and Rotwein, 1989; LeRoith *et al.*, 1992b). Both of the IGFs are essential to embryonic development (Baker *et al.*, 1993), and nanomolar concentrations of both are maintained in the circulation into adult life. Afterbirth, however, IGF-I appears to have the predominant role in regulating growth, whereas the physiologic role of IGF-II is unknown.

In the pancreas, increased IGF-I expression is localized to focal areas of regeneration in pancreatectomized rats and dogs (Smith *et al.*, 1991; Hayakawa *et al.*, 1996), suggesting that IGF-I contributes to the growth or differentiation of pancreatic tissue. Furthermore, it has been drescribed that islet cells contain IGF-I receptors (Van Schravendijk *et al.*, 1987). Mice lacking IGF-I receptor and insulin receptor substrate-2 (IRS-2) show marked reduction of ß-cell mass and die from diabetes due to ß-cell insufficiency (Van Schravendijk *et al.*, 1987), indicating that IGF-I receptors couple to IRS-2 in the pancreatic islet to mediate ß-cell development, proliferation and survival. In addition, IGF-I increases ß-cell proliferation *in vitro*, which is dependent on IRS-mediated induction of phosphatidylinositol 3'-kinase (PI3-kinase) (Hugl *et al.*, 1998).

IGF-I is also considered a survival factor that has a widespread antiapoptotic effects on many death signals. The main signaling pathway for IGF-I receptor–mediated protection from apoptosis originates with the interaction of the IGF-I receptor with the IRS-1 (Ruderman *et al.*, 1990), leading to activation of PI3-kinase and Akt/protein kinase B (Kauffmann-Zeh *et al.*, 1997), and the phosphorylation and inactivation of BAD (Datta *et al.*, 1997), a member of the Bcl-2 family of proteins. The IGF-I receptor also activates alternative pathways for protection, one through activation of mitogen-

activated protein kinase (MAPK) (Peruzzi *et al.*, 1999) and another through the activation of Raf-1 and its translocation to the mitochondria (Peruzzi *et al.*, 1999). All three pathways result in BAD phosphorylation. The presence of multiple antiapoptotic pathways may explain the remarkable efficiency of the IGF-I receptor in protecting cells from apoptosis. Thus, it has been shown that IGF-I treatment protects islets against cytokine-mediated inhibition of insulin secretion, stimulation of nitric oxide formation, and cell death by apoptosis (Mabley *et al.*, 1997; Castrillo *et al.*, 2000; Storling *et al.*, 2005). Similarly, adenoviral gene transfer of IGF-I to human islets in vitro prevents IL-1ß–mediated nitric oxide formation and IL-1ß–induced, Fas-mediated apoptosis (Giannoukakis *et al.*, 2000a). The anti-inflammatory and antiapoptotic role of IGF-I in pancreatic ß-cells is dependent on PI 3-kinase activation (Storling *et al.*, 2005). In addition, before diabetes onset, subcutaneous administration of recombinant IGF-I to young nonobese diabetic (NOD) mice reduces diabetes incidence and pancreatic insulitis (Bergerot *et al.*, 1995; Kaino *et al.*, 1998)(31, 32). In NOD mice injected with autoreactive T cells from diabetic NOD mice, IGF-I treatment reduces massive T cell invasion of islets and delays the onset and decreases the incidence of diabetes (Bergerot *et al.*, 1995).

7.2. Transgenic mice expressing IGF-I in ß-cells.

Patients of type 1 diabetes are identified after diabetes onset when ß-cell destruction is nearly complete. ß-cell regeneration from islet cell precursors might reverse type 1 diabetes. However, identification of factors that induce ß-cell neogenesis and replication and prevent autoimmune destruction of new islets is required.

In our laboratory we have previously generated transgenic mice expressing IGF-I specifically in ß-cells by using the rat insulin promoter-I in both C57Bl6/SJL and CD1

genetic backgrounds. We observed that the expression of IGF-I counteracts cytotoxicity and insulitis after treatment with multiple low doses of STZ. All STZ-treated control mice developed high hyperglycemia (>600mg/dl), hypoinsulinemia, lost body weight and died. In contrast, STZ-treated C57Bl6/SJL transgenic mice showed mild hyperglycemia (about 300md/dl) for about one month, but gradually normalized glycemia and had normal survival. However, STZ-treated transgenic mice of CD-1 genetic background, which is highly susceptible to low doses of streptozotocin, developed high hyperglycemia (>600 mg/dl), hypoinsulinemia, polydipsia and polyfagia, but gradually normalized all metabolic parameters and survived for longer than 8-months. This was paralleled with increased ß-cell mass through neogenesis and ß-cell replication. Expression of IGF-I did not affect other organs since control and transgenic mice had similar serum IGF-I, glucose and insulin levels and body weight, consistent with an autocrine/paracrine role of IGF-I.

We examined whether IGF-I also protects islets from autoimmune destruction (Casellas et al., manuscript submitted). To this end, we used transgenic mice expressing IFN-ß in ß-cells. These mice hyperexpressed islet $ß_2$-microglobulin and Fas, and increased lymphocytic infiltration. In addition, pancreatic islets showed high insulitis and these mice developed overt diabetes when treated with very low doses of streptozotocin (STZ), which did not affect control mice. Expression of IGF-I in IFN-ß-expressing ß-cells of double transgenic mice reduced $ß_2$-microglobulin expression and counteracted islet infiltration. This was parallel to a decrease in ß-cell death by apoptosis in islets of STZ-treated IGF-I+IFN-ß-expressing mice. These mice were normoglycemic and normoinsulinemic, and presented similar pancreatic insulin levels as healthy mice. Thus, local expression of IGF-I prevented islet infiltration and ß-cell death in mice with increased susceptibility to diabetes (Casellas et al., manuscript

submitted). These results indicate that pancreatic expression of IGF-I may regenerate and protect ß-cell mass, and suggest that IGF-I gene transfer to pancreas might be a suitable therapy to reverse type 1 diabetes.

III. OBJECTIVES

We have previously demonstrated that transgenic mice expressing IGF-I in ß-cells recovered from STZ-induced diabetes. The main objective of the present study were (1) to further investigate the mechanism by which IGF-I regenerate the pancreas; (2) to develop new gene transfer approaches to the pancreas *in vivo* by using viral vectors trying to design a new gene therapy approach for type 1 diabetes.

The specific aims of this work are:

1.- To study whether IGF-I expression in ß-cells may recruit bone marrow derived cells to the islets and whether these cells may contribute to the regeneration of the endocrine pancreas after treatment with STZ.

2.- To develop new gene transfer approaches using viral vectors to transduce the pancreas *in vivo*.

2.1 Gene transfer approaches to murine pancreas using viral vectors

2.2 Gene transfer approaches to canine pancreas using viral vectors

IV. RESULTS AND DISCUSSION

PART I:

CHARACTERIZATION OF BONE MARROW-DERIVED CELL FATE IN THE PANCREAS

INTRODUCTION

Successful reversal of type 1 diabetes requires ß-cell regeneration. We have previously shown that specific expression of IGF-I in ß-cells of transgenic mice induces ß-cell replication and neogenesis, and allows transgenic mice to recover ß-cell mass and counteract hyperglycemia after streptozotocin (STZ) treatment (George *et al.*, 2002). It has also been shown that IGF-I delivery to skeletal muscle acts as a powerful enhancer of the muscle regeneration response and is able to augment recruitment of bone marrow cells to sites of muscle damage (Musaro *et al.*, 2004). The use of stem cells, a potential renewable source of pancreatic ß-like cells is an attractive goal and is currently under investigation by many groups. BM is an important source of easily procurable adult stem cells, and cells derived from bone marrow compartment have shown to possess pluripotent capabilities (Herzog *et al.*, 2003). In addition, the use adult stem cells may solve the ethical issues surrounding embryonic stem cells. Therefore, differentiation of BM cells into functional ß-cells may represent an attractive source for cell replacement therapy for type 1 and type 2 diabetes. One study found a significant contribution of BM-derived cells to the ß-cell compartment, and one month after BM transplantation about 1.7-3% of insulin-producing cells were from donor origin (Ianus *et al.*, 2003). In addition, injection of BM cells into diabetic recipients results in pancreatic regeneration and reduction of hyperglycemia (Hess *et al.*, 2003). In contrast with these initial reports, subsequent work has shown little evidence of BM transdifferentiation into ß-cells in murine healthy pancreas or after pancreatic damage *in vivo* (Choi *et al.*, 2003; Lechner *et al.*, 2004; Taneera *et al.*, 2006). In addition, an elegant study using genetic labeling of ß-cells showed that maintenance of adult ß-cell mass in adult mice is due to

replication rather than stem cell differentiation, both in healthy mice as well as after pancreatectomy (Dor *et al.*, 2004). Thus, the capacity of BM to differentiate into ß-cells remains controversial. It has been suggested that experimental conditions, such as the bone marrow transplantation protocol, the method used for the identification of cell phenotype, and the model of pancreatic damage might explain these differences. Therefore, new studies are required to better understand the potential of BM-cells for differentiate into ß-cell and subsequently for the treatment of diabetes.

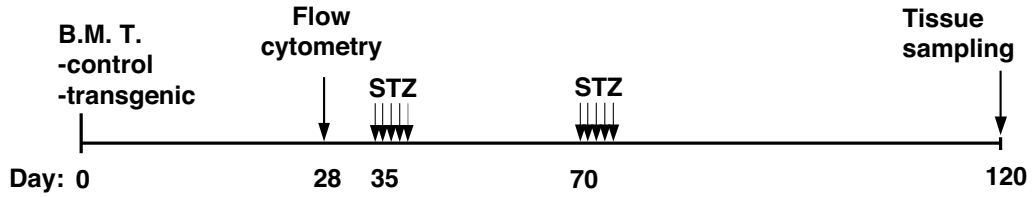
Here we transplanted BM from GFP transgenic mice into IGF-I transgenic and wild type mice to study the contribution of BM-derived cells to the exocrine and endocrine pancreas.

1. BONE MARROW CELL TRANSPLANTATION IN TRANSGENIC MICE OVEREXPRESSING IGF- I IN ß-CELLS.

The contribution of bone marrow derived cells (BMC) to endocrine pancreas regeneration in streptozotocin (STZ)-treated transgenic mice expressing IGF-I in ß-cells was examined. To this end, femur and tibia BMC were isolated from donor transgenic mice, expressing green fluorescent protein (GFP) under the control of the ubiquitous chicken ß-actin promoter. These cells (10^7) were transplanted by tail vein injection into lethally irradiated recipient wild type and transgenic mice. Figure 1A summarizes the experimental design of this study. To examine the efficiency of BMC engraftment, twenty-eight days after cell transplant, nucleated GFP-expressing cells in peripheral blood were measured by flow cytometry. In all mice, a high percentage of chimerism (>85%) was observed (Fig. 1B), and this percentage was maintained 4-months after transplant (data not shown). Furthermore, immunohistochemical analysis of pancreatic lymph nodes revealed that recipient immune cells were replaced by donor GFP-expressing cells (Fig. 1C).

In addition, high expression of IGF-I was detected in islets from transgenic mice four months after transplant, indicating that the expression of the transgene was not altered by the transplantation protocol (Fig. 2).

A



B

Chimerism in peripheral nucleated blood cells

	Female	Male
Con	86,8 ± 1,2 (n=8)	84,7 ± 2,3 (n=8)
Tg	86,5 ± 0,9 (n=8)	86,9 ± 2,4 (n=8)

C

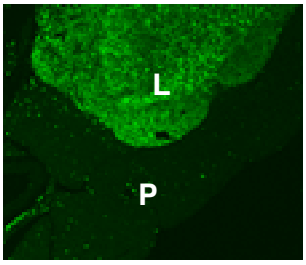


Figure 1. Bone marrow transplantation into transgenic mice expressing IGF-I in ß cells. (A) Schematic representation of the experimental design used for bone marrow transplantation (BMT). Female and male wild type mice (n=8, per group), and female and male IGF-I transgenic mice (n=8, per group) were lethally irradiated and received transplants of BMC from GFP transgenic mice. Twenty eight days after the transplant, chimerism in nucleated peripheral blood cells were measured by flow cytometry. After 35 days the first STZ (30mg/kg, 5 consecutive days) treatments were given to 50% of the mice from each group, and the second injections of STZ (40 mg/kg, 5 consecutive days) were given to the same animals one month later. All mice were sacrificed 4 months after the BM transplantation and pancreata were analyzed for chimerism. **(B)** Percentage of GFP positive cells in peripheral nucleated cells measured by FACS analysis. Results are expressed as mean ± SE (n=8 per group). **(C)** Pancreatic lymph node (L) expressed GFP four months after the BMT. P=pancreas. Original magnification 40x.

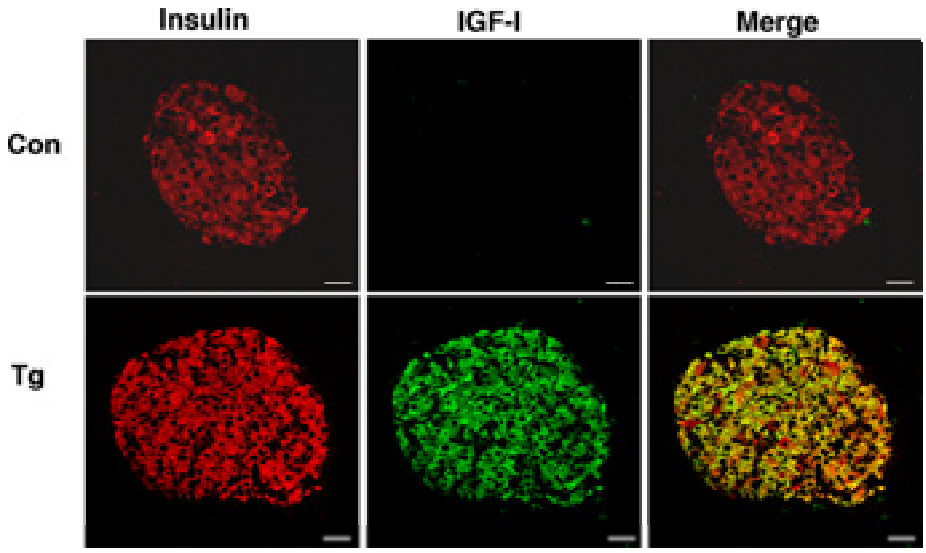


Figure 2. Immunohistochemical analysis of insulin and IGF-I in control and transgenic islets. Scale bars: 23µm (con) and 27 µm (Tg).

2. COUNTERACTION OF DIABETES IN BMC TRANSPLANTED IGF-I TRANSGENIC MICE.

One month after BMC transplantation, 50% of wild-type and 50% of IGF-I transgenic mice were treated with STZ (30 mg/kg b.w.) for 5 consecutive days. Thirty days thereafter, wild-type mice were hyperglycemic while blood glucose levels did not increase in STZ-treated IGF-I transgenic mice, which maintained normoglycemia (Fig. 3). This suggested that IGF-I expression protected ß-cells against STZ damage. Therefore, to achieve destruction of ß-cells in transgenic mice, a second treatment with higher doses of STZ (5x40 mg/Kg b.w.) was administered to the animals that previously received STZ. Blood glucose levels continued to rise in wild-type mice (over 500 mg/dl). However, although transgenic mice developed hyperglycemia (about 400 mg/dl) by 30 days after STZ-treatment, blood glucose levels gradually decreased, suggesting that, as previously observed (George *et al.*, 2002), an IGF-I-mediated regeneration process occurred in these mice (Fig. 2*A*). Non-STZ treated wild-type and transgenic mice maintained normoglycemia during the whole study (Fig. 3).

Three months after STZ treatment, and 4-months after the transplant, all groups of mice were sacrificed. Double insulin and glucagon immunostaining of non-STZ treated wild-type (Con) and IGF-I transgenic (Tg) pancreas showed islets with normal distribution of insulin-expressing cells in the core and glucagon-expressing cells in the periphery (Fig. 4). Diabetic wild-type mice (STZ-Con) showed islet destruction and lack of insulin-expressing cells. However, STZ-treated transgenic mice showed large islets with altered distribution of α and ß cells, since α-cells no longer formed a mantle

around the ß-cell core. These results suggested that in STZ-treated transgenic mice ß-cells were probably destroyed and regenerated, leading to disorganization of islet cell distribution (Fig. 4).

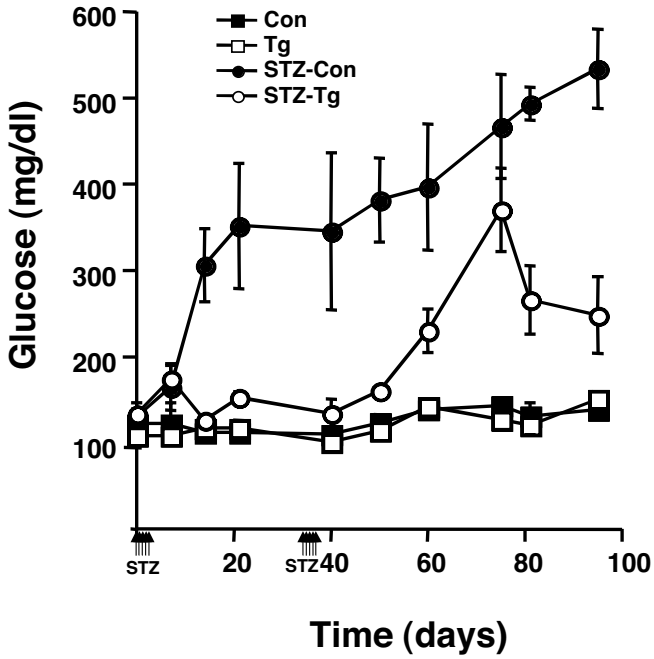


Figure 3. Blood glucose levels after STZ-treatment. (A) Blood glucose levels of control (Con) and transgenic (Tg) mice treated with or without STZ.

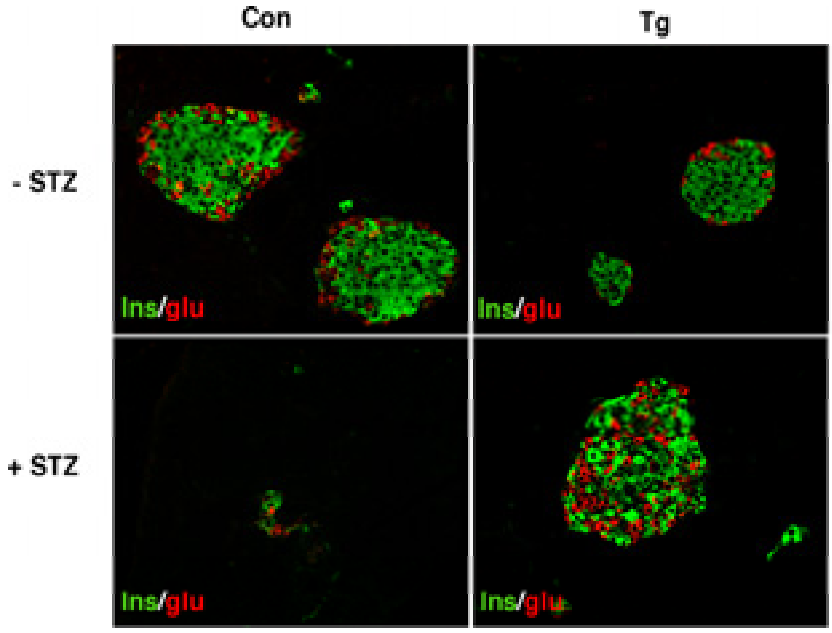


Figure 4. Pancreatic islet structure after STZ-treatment. Islet architecture in mice treated with (+STZ) or without STZ (-STZ). Immunohistochemical analysis of insulin (green) and glucagon (red) expression in control and transgenic mice is shown. Original magnification x200.

3. CONTRIBUTION OF BMC TO ENDOCRINE PANCREAS.

The contribution of BMC to the endocrine pancreas compartment of recipient mice was analyzed by double GFP and insulin immunostaining. After examination of more than 900 islets in the four groups of mice (wild-type and transgenic mice, treated and non-treated with STZ) only a total of 5 double GFP^+/$insulin^+$ cells were detected (Fig. 5a-d) and Table 1. This indicated that islet production of IGF-I did not recruit BMC for differentiation into ß-cells. However, GFP^+ cells were observed inside the islets (Fig. 5e-h). When these cells were counted, no significant differences were detected between the four groups of mice (Table 1), suggesting that recruitment of GFP^+ cells was not dependent on IGF-I expression nor of STZ treatment. In addition, we have also carefully examined single insulin positive cells (>100 cells) scattered in the parenchyma or located near the ducts, and found no double GFP^+/$insulin^+$ cells (Fig. 5i-l).

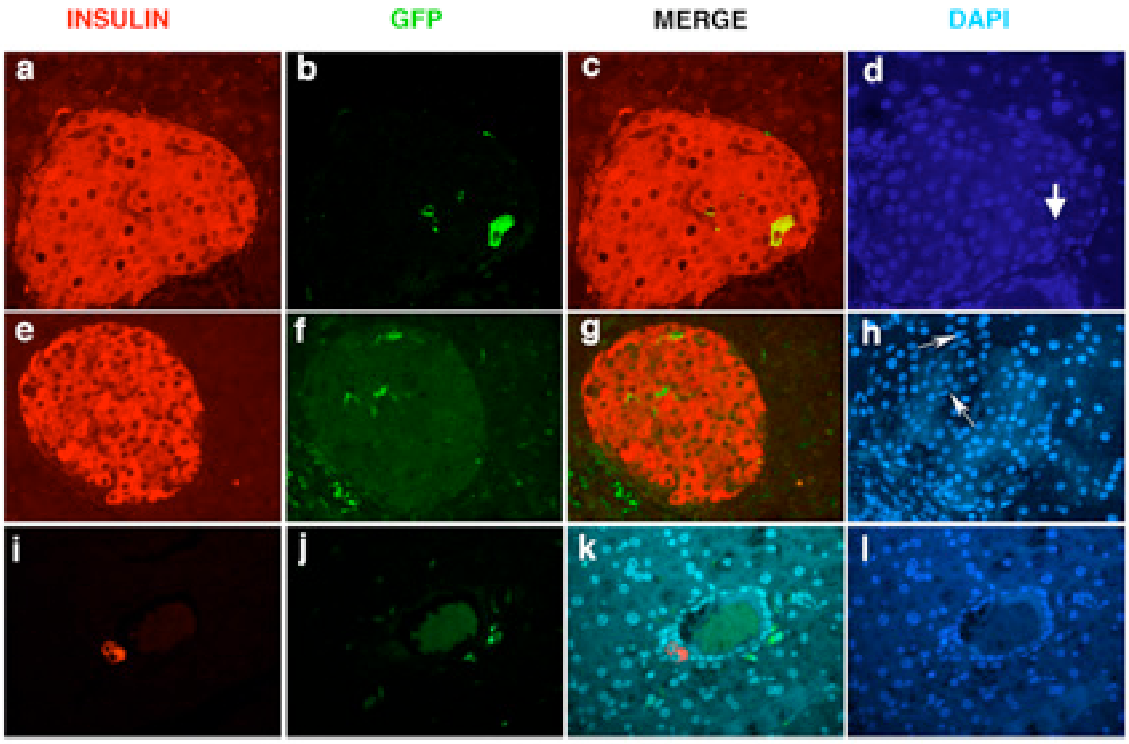


Figure 5. Contribution of BMC to endocrine pancreas. Immunohistochemical analysis of GFP (green) and insulin (red) expression and nuclei (blue) in islets. Representative images of insulin and GFP double positive cell (a-d), GFP positive cells inside the islet (e-h) and insulin positive but GFP negative cells near the duct (i-l) are shown. Original magnification x630 (a-d) and x400 (e-l).

	Total islets counted		Total double Ins^+/GFP^+ cells		GFP^+ cells inside the islet / islet	
	M	**F**	**M**	**F**	**M**	**F**
Con	106	140	0	1	0.6±0.03	0.8 ±0.18
STZ-Con	50	60	0	0	1.5±0.67	1.2 ±0.24
TG	184	208	1	2	0.63±0.06	1.5 ±0.34
STZ-TG	98	68	1	0	0.57±0.17	1.07 ±0.8

Table 1. Quantification of GFP^+ cells within the endocrine pancreas. Double immunohistochemical analysis of GFP and insulin was performed in two sections per animal and four animals per group. All the islets in these sections were counted. Cells that were double positive for GFP and insulin immunostaining were counted as double GFP^+/Ins^+. All the cells that were GFP positive but insulin negative inside the islets were counted and were divided by the total number of islets. Values are expressed as the mean±SEM of GFP^+ cells per islet.

To determine whether BMC maintained the hematopoietic phenotype, expression of the panhematopoietic marker CD45 in islets was analyzed. Double immunohistochemical analysis showed that GFP-expressing cells in the islets also expressed the CD45 marker (Fig. 6), indicating a hematopoietic phenotype of these cells.

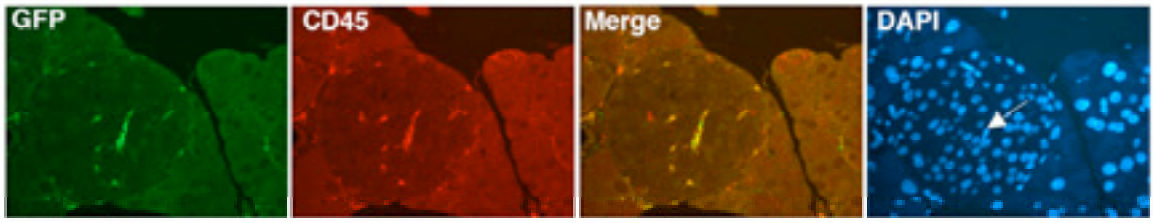


Figure 6. Analysis of CD45 expression in BMC within the endocrine pancreas. Double immunohistochemical analysis showed that GFP positive cells (green) were also positive for the panhematopoietic marker CD45 (red). Original magnification x400.

Furthermore, three months after STZ treatment, IGF-I expression was maintained in islets of transgenic mice and correlated with the expression of insulin (Fig. 7). This was consistent with the fact that the insulin producing cells originated from preexisting IGF-I-expressing ß-cells.

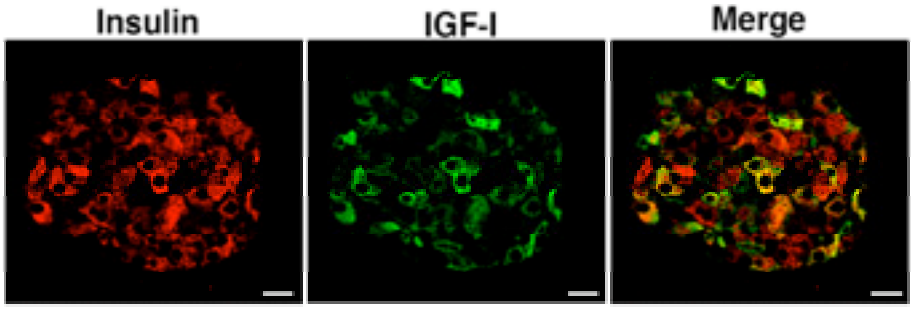


Figure 7. IGF-I expression in transgenic islets. Immunohistochemical analysis showed that three months after STZ treatment, IGF-I expression (green) was maintained in islets of transgenic mice and correlated with the expression of insulin (red). Scale bars: 17.5µm

4. CONTRIBUTION OF BMC TO EXOCRINE PANCREAS.

The contribution of BMC to the exocrine pancreas was also analyzed by amylase and GFP double immunostaining of pancreatic sections. GFP-expressing cells were randomly distributed in the interstitium of the exocrine pancreas, but these cells did not present acinar morphology (Fig. 8A). Only one double amylase/GFP positive cell was observed out of 32 pancreatic sections of the four groups of animals studied (data not shown). Cells co-expressing GFP and the CD45 panhematopietic marker were localized in the interstitial space of the exocrine pancreas of healthy wild-type mice (Fig. 8B), indicating that the presence of BMC is most likely a natural process. In addition, similar percentages of interstitial GFP-expressing cells were found in healthy IGF-I transgenic mice, and in STZ-treated wild type and transgenic mice (data not shown).

To investigate whether BMC contributed to blood vessel formation in the exocrine pancreas and thus the presence of GFP positive cells in pancreatic small arteries, GFP/α-smooth muscle actin (α-SMA) double immunostaining of pancreas sections was performed. Arteries showed higher α-SMA staining of the wall than veins because of the increased amount of smooth muscle cells present in the tunica media (Fig. 9A).

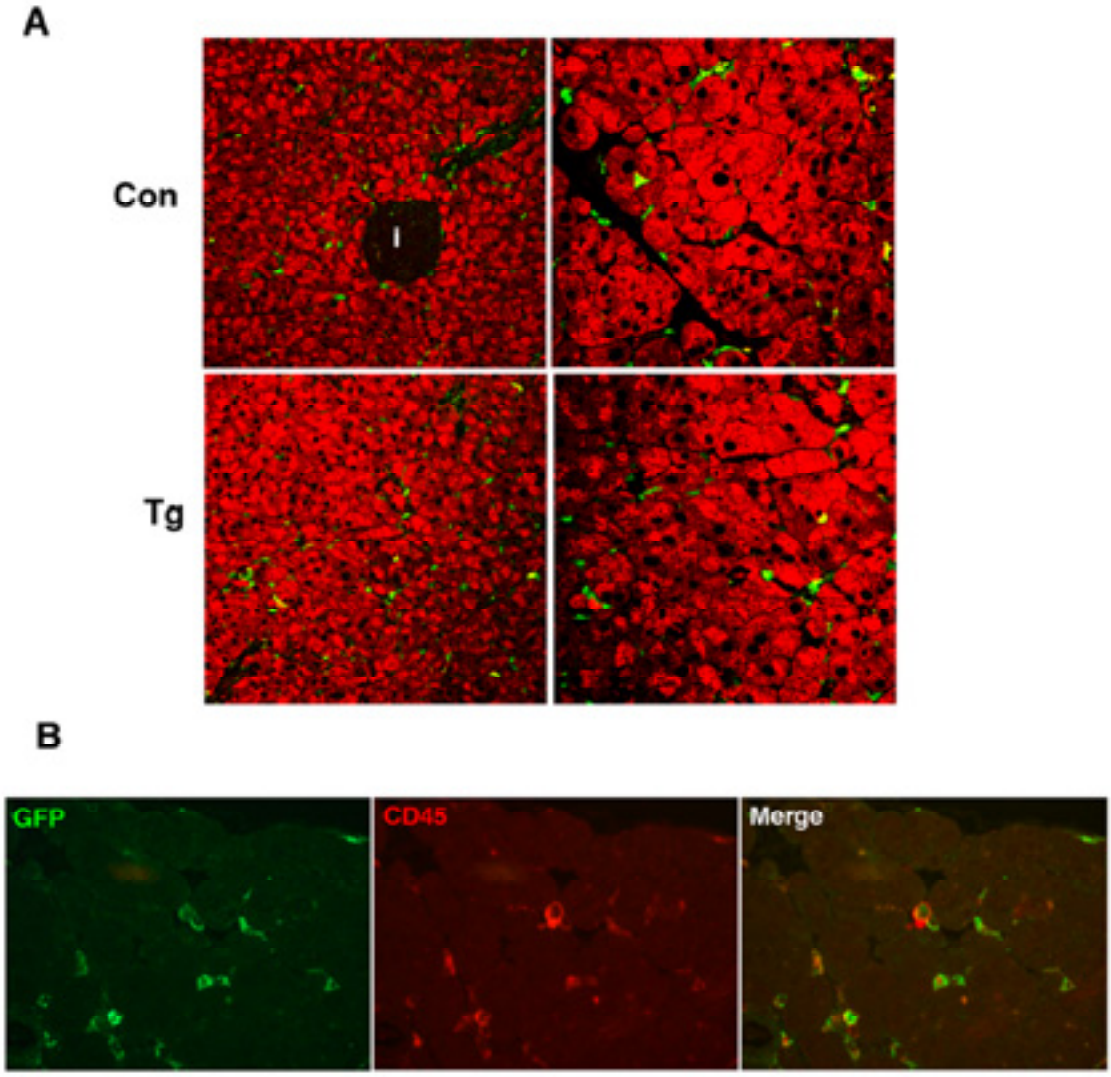


Figure 8. Contribution of bone marrow derived cells to exocrine pancreas. (A) Bone marrow-derived cells (green) were located mainly in the interstititum of the exocrine tissue, as demonstrated using double immunolabelling of GFP (green) and amylase (red), a marker of acinar cells. Original magnification x100 (a,c), x400 (b,d). (**B**) BM-derived cells (green) localized in the interstitium of exocrine tissue expressed the panhematopoietic marker CD45 (red). Original magnification x400.

In the pancreas of all four groups of mice, GFP-expressing cells surrounding the main blood vessels and ducts were detected. However, despite the presence of GFP cells in close proximity of small arteriole, no $GFP^+/\alpha\text{-}SMA^+$ cells were detected in the tunica media of vessels. Moreover, GFP^+ cells were never found adjacent to the α-SMA positive cells on luminal side of the arteries, indicating that BMC did not contribute to the endothelial cell layer (Fig. 9A). In addition, some GFP^+ cells were found adjacent to

vessels in islet periphery, but none of these cells were incorporated in blood vessel wall (Fig. 9B).

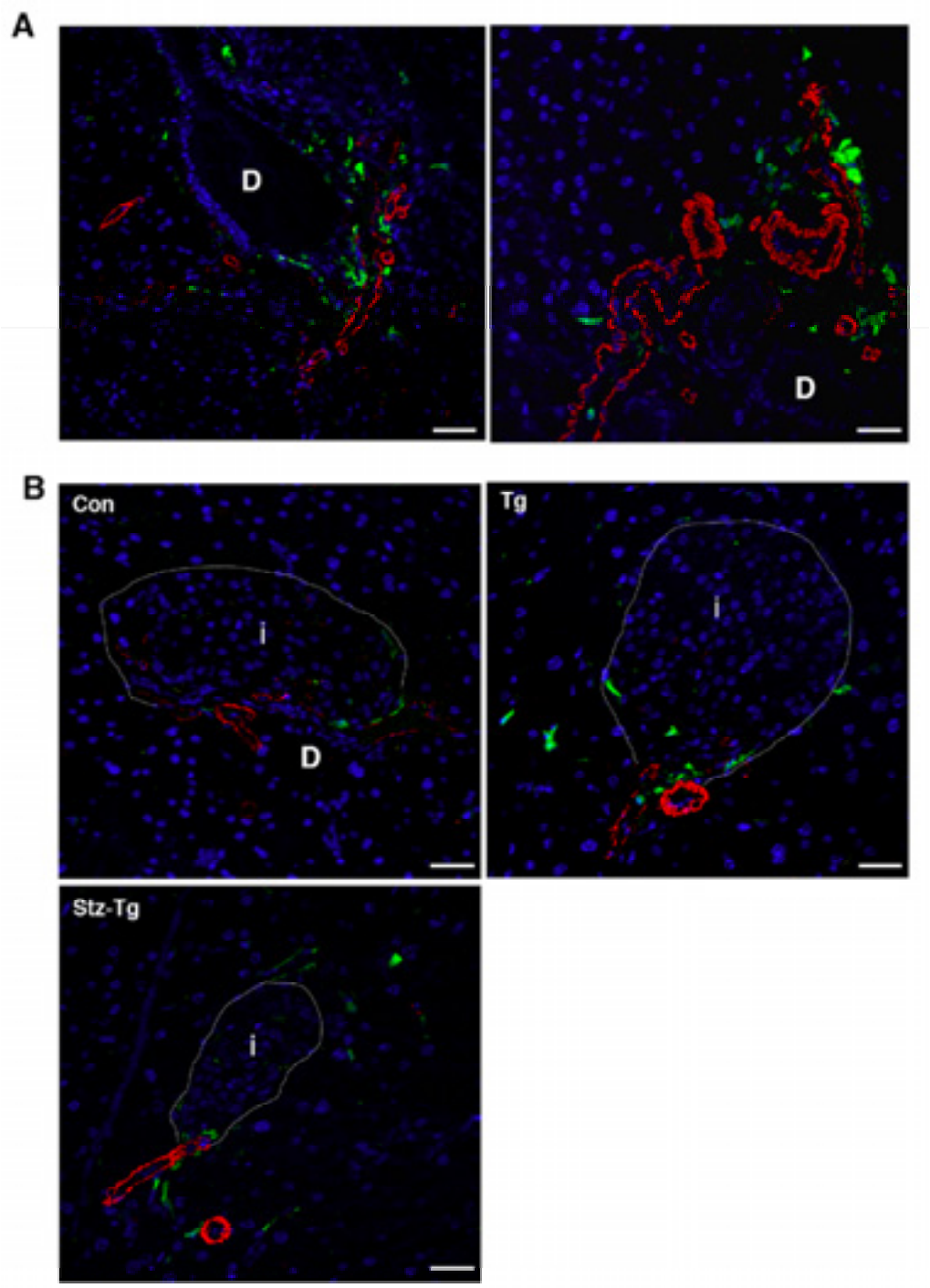


Figure 9. BM-derived cells and blood vessels. (**A**) Main blood vessels in the pancreas were immunostained using the α-SMA antibody (red) and groups of GFP^+ cells (green) were present surrounding the blood vessels (red) and pancreatic ducts (D). Scale bars: 37.5 µm (left), 22 µm (right). (**B**) GFP expressing cells were found around the islet (i) and were not limited to the islet-associated α-SMA^+ blood vessels (red). Scale bars: 26 µm (con), 25 µm (Tg and STZ-Tg).

DISCUSSION

Bone marrow differentiation into ß-cell may represent a very attractive approach for cellular replacement therapy in type 1 diabetes. However, to date, very contradictory results have been obtained studying the feasibility of BMC differentiation into ß-cells *in vivo* when transplanted into mice recipients (Choi *et al.*, 2003; Hess *et al.*, 2003; Ianus *et al.*, 2003; Lechner *et al.*, 2004; Taneera *et al.*, 2006). Since BM stem cells are easily procurable and allow autologous transplantation, they could be considered as an unlimited source of ß-cells and, therefore, this differentiation event needs to be further investigated. Here we have transplanted whole BM from transgenic mice expressing GFP ubiquitously into lethally irradiated wild type and IGF-I expressing mice. Hematopoietic chimerism in peripheral blood was >85% in all transplanted mice, and four months after the transplant, pancreatic lymph nodes were reconstituted by GFP^+ cells indicating that BM engraftment was very efficient. Four month after the transplant, healthy wild type mice showed BMC mainly in the interstitium of the exocrine and endocrine pancreas, and these cells expressed the panhematopoietic marker CD45 indicating that differentiation had not occurred. We found only one cell expressing GFP and insulin after the analysis of more than 10000 ß-cells, which means less than 0.01%. The percentage of transdifferentiated ß-cells reported by Ianus et al. 4-6 weeks after the transplant was about 1.7-3% (Ianus *et al.*, 2003). Since we sacrificed the mice 4 months after BM transplantation, we should expect >6% of ß-cells derived from BM, based upon their results, and our results were largely below this percentage. In addition to our observations, other groups have reported little or no differentiation of BM to ß-cells *in vivo* (Choi *et al.*, 2003; Lechner *et al.*, 2004; Taneera *et al.*, 2006). However,

several studies have suggested that the ability of BMC to change the hematopoietic phenotype *in vivo* may be dependent of tissue-specific damage (Grompe, 2003; Hess *et al.*, 2003; Orlic *et al.*, 2003; Musaro *et al.*, 2004). To investigate this, we induced experimental diabetes by STZ injection. Three months after diabetic wild type mice showed similar amount of BMC in the whole pancreas, and similar numbers of GFP^+ cells surrounding residual islets. In addition, no GFP^+/$insulin^+$ double positive cells were found and virtually all donor cells were GFP^+/$CD45^+$. These data indicated that STZ-induced damage did not increase the recruitment of BMC to the pancreas. The injection of a certain population of BMC has been shown to reduce the hyperglycemia in diabetic mice (Hess *et al.*, 2003), however, in this study STZ injection was prior to the BM transplantation and this might explain the difference observed in our experiments. The appearance of GFP^+/$insulin^+$ cells might not only be due to transdifferentiation, but also to cell fusion events, as has been clearly demonstrated in the liver (Wang *et al.*, 2003a), and might also be the origin of the double GFP^+/Ins^+ cells observed in our experiments.

IGF-I is a powerful enhancer of the regeneration response in the muscle, and mediates the recruitment of bone marrow cells to sites of tissue damage and augments local repair mechanisms (Musaro *et al.*, 2004). In addition, delivery of IGF-I by three different methods (plasmid electroporation, injection of genetically engineered myoblasts and recombinant protein injection) increased the integration of BMC up to four-fold in the muscle (Sacco *et al.*, 2005). We have previously shown that transgenic mice expressing IGF-I in ß-cells recover from type 1 diabetes by a mechanism involving replication and neogenesis of ß-cells (George *et al.*, 2002). To study whether BMC may also contribute to this process we transplanted IGF-I transgenic mice with whole BM

from GFP transgenic mice following the same protocol of wild type mice. Peripheral blood chimerism, the amount of BMC in the whole pancreas, and the number of GFP^+ cells surrounding the islets were not significantly different to wild type mice. In addition, we found only three double positive GFP^+/Ins^+ cells after analysis of more than >390 islets, futher indicating the reduced ability of BMC to transdifferentiate *in vivo*, even when a well known ß-cell growth factor is expressed. In this study, the chimerism in the pancreas was studied in 6-month old transgenic mice. At this age, we have previously shown that IGF-I transgenic mice in C57Bl6/SJL background developed mild islet hyperplasia (George *et al.*, 2002). Therefore, these data suggest that BMC do not contribute to ß-cell expansion in this transgenic model, and also agree with the fact that adult pancreatic ß-cells are formed by self-duplication rather than stem-cell differentiation (Dor *et al.*, 2004). To induce a destruction and regeneration process in the pancreas of IGF-I transgenic mice we treated them with STZ. Although in our previous study (George *et al.*, 2002) we treated mice with 50 mg/kg STZ, here we injected a lower dose of 30 mg/kg because these mice were challenged with lethal irradiation and BM transplantation. Interestingly, in contrast to wild type mice, we observed that transgenic mice did not developed hyperglycemia when treated with this STZ dose. Therefore, one month after the first STZ injection, a second dose of 40 mg/kg was given to wild type and transgenic mice and only then we observed an increase in the glycemia of transgenic mice, indicating ß-cell destruction. However, these mice progressively reduced the glycemia suggesting ß-cell regeneration. Indeed, islets from STZ-treated transgenic mice were larger than STZ-treated wild type mice, and showed altered distribution of α and ß cells suggesting that some destruction-regeneration of ß-cell might occurred. We counted

GFP^+/Ins^+ in the islets of these mice 3 months after the injection of STZ and found only one cell, suggesting that BMC do not contribute to the regeneration of ß-cells in IGF-I transgenic mice.

The possibility that BMC might be incorporated into blood vessels have been well studied during last few years, and controversial results have been obtained (Asahara *et al.*, 1997; Asahara *et al.*, 1999; De Palma *et al.*, 2003; Machein *et al.*, 2003; Walter *et al.*, 2004; Ziegelhoeffer *et al.*, 2004). In the pancreas, the study of Hess et al. suggested that BMC might differentiate into endothelial cells within the islet capillaries and secrete factors that might contributed to the improvement of ß-cell function and the reduction of diabetic hyperglycemia (Hess *et al.*, 2003). This study used the CD31 marker to identify the BMC that had differentiated into an endothelial phenotype. However, this marker has also been shown to label myeloid cells (Geissmann *et al.*, 2003; Curat *et al.*, 2004; Rohde *et al.*, 2006). It is possible that double positive $GFP^+/CD31^+$ were not endothelial cells, but still hematopoietic cells. Another report found that BMC expressed vonWillebrand factor, an endothelial marker (Mathews *et al.*, 2004). However, in this study no benefits to the BM-transplanted diabetic mice were observed compared with non-transplanted diabetic mice (Mathews *et al.*, 2004). Here in this study, we have used the α-smooth muscle actin (α-SMA) marker to detect blood vessels and we found that groups of BMC were present near the main ducts and blood vessels of the pancreas. However, these cells did not participate actively in the formation of the vessel, nor in the exocrine pancreas neither in the islets. These results agree with recent observation showing that although BMC are attracted to sites of neovascularization they do not contribute to vessel formation (Zentilin *et al.*, 2006).

Although Zentillin *et al*. reported very few BMC that were $CD31^+$ using the Tie2-GFP reporter system (in which activation of GFP expression only occurs in endothelial cells), the authors were unable to confirm the presence of BMC neither in the endothelium of small vessels, nor in larger collateral arteries. Furthermore, most of the recruited cells were positive for the myelo/monocytic markers CD11b and CD45 (Zentilin *et al.*, 2006). Lastly, it has been shown by others that the BMC in the pancreas expressed the myeloid antigen Mac-1/Gr-1, whereas these cells did not express T-cell (CD4, CD8) and B-cell (B220) markers (Taneera *et al.*, 2006).

We have demonstrated that *in vivo* transdifferentiation of BMC is an extremely rare and inefficient event. Approaches based on this phenomenon are unlikely to be used for ß-cell replacement in diabetes in the near future. In addition, we have observed that IGF-I did not increase the recruitment of BM-derived cells to the islets and these cells did not contribute to the regeneration observed in these mice after treatment with STZ.

PART II:

IN VIVO GENE TRANSFER TO MURINE AND CANINE PANCREAS

INTRODUCTION

To genetically engineer the endocrine pancreas the selection of both the vector and the route of administration are key and need to be further studied. Several vectors can be used to transfer foreign genes to pancreatic islets and to pancreatic ß-cell lines *in vitro*. Among them, adenovirus show ß-cell tropism and high transduction efficiency (Becker *et al.*, 1994; Csete *et al.*, 1995; Benhamou *et al.*, 1996; Saldeen *et al.*, 1996; Muruve *et al.*, 1997; Smith *et al.*, 1997; von Herrath *et al.*, 1997; Weber *et al.*, 1997; Judge *et al.*, 1998; Yasuda *et al.*, 1998; Giannoukakis *et al.*, 1999b; Grey *et al.*, 1999a; Grey *et al.*, 1999b; Guo *et al.*, 1999; Uchikoshi *et al.*, 1999; Giannoukakis *et al.*, 2000a; Giannoukakis *et al.*, 2000b; Moriscot *et al.*, 2000; Contreras *et al.*, 2001a; Contreras *et al.*, 2001b; Alexander *et al.*, 2002; Bertera *et al.*, 2003). Direct pancreatic injection and retrograde pancreaticobiliary duct delivery of adenovirus can transduce the exocrine pancreas, but these approaches induce severe inflammation and toxicity (Raper and DeMatteo, 1996; McClane *et al.*, 1997a; McClane *et al.*, 1997b; McClane *et al.*, 1997c; Wang *et al.*, 2004a). Systemic delivery of adenovirus fails to infect pancreas *in vivo*, because the liver rapidly removes them from circulation (Ye *et al.*, 2000). Injection of adenovirus to the blood stream after a temporary closure of the portal vein, hepatic artery and bile duct (portal clamp) results in increased concentration of circulating virus during the clamp. This allows adenovirus to infect other organs such as intestine, kidney and lungs (Ye *et al.*, 2000). Here we examine new gene transfer approaches to deliver genes to the pancreas *in vivo* by systemic delivery of adenovirus in mice with clamped hepatic circulation. Several adenoviral vectors carrying marker genes under the control of ubiquitous (CMV) or selective (insulin) promoters has been used to detect the

transduced cells. In addition to adenoviral vectors, we have studied the ability of adeno-associated viral vectors to transduce the pancreas using different routes of administration. AAV vectors were injected either intraperitoneally, systemically or directly into pancreatic ducts and transduction efficiency was analyzed.

Beagle dogs have been used both as large animal models of many human diseases and in several gene therapy approaches, including hemophilia and retinal degeneration (Snyder *et al.*, 1999; Acland *et al.*, 2001; Chuah *et al.*, 2003; Arruda *et al.*, 2004a; Arruda *et al.*, 2004b). However, gene transfer to canine pancreas has not yet been reported. Taking into account the anatomy and vascularization of the dog pancreas, we hypothesized that a clamp of pancreatic circulation could be applied *in situ* and vectors injected directly into the pancreatic vessels. This could potentially increase pancreas transduction and avoid liver damage. Here we have examined the ability of adenoviral vectors to transduce the canine pancreas using this approach. In addition to healthy dogs, we have also examined if this approach could be used in a diabetic dog.

1. IN VIVO GENE TRANSFER TO MURINE BETA CELLS BY USING VIRAL VECTORS.

In this study we have developed a new approach for pancreatic gene transfer by systemic injection of adenoviral vectors in mice with clamped hepatic circulation. In contrast to direct or ductal injection, systemic delivery results in homogeneous distribution of the vector within the pancreas and the islets.

1.1 Gene transfer to murine pancreas in vivo using first generation adenoviral vectors.

1.1.1. Experimental design used for adenovirus administration to mice.

Adenoviral vectors possess many advantages as gene delivery systems. These vectors have a broad cell tropism, they can transduce both quiescent and dividing cells, and high titers can be obtained easily in the lab (see introduction section 5.2.1). In addition, Ad vectors have been shown to be very efficient at transducing islets and ß-cells, a feature that makes these vectors an attractive delivery system for pancreas (see introduction section 6.3.1). However, when Ad are injected into the circulation, the liver removes them very quickly. Hence, high transduction of hepatocytes and Kupffer cells can be observed, whereas the vector transduces very few extra-hepatic cells. Here we have studied whether clamping the hepatic circulation and systemic injection of the vector may result in increased transduction of the pancreas. To this end, mice were anesthetized and abdominal laparatomy was performed. The hepatic triad, including portal vein, hepatic artery and bile duct, was closed by using a microvascular clamp. Immediately, Ad vectors were injected into the jugular vein and the clamp was

maintained for 30 minutes. Afterwards, the clamp was opened and abdominal wall was sutured. Mice were sacrificed at different times to study tissue transduction. The experimental procedure is shown in Fig. 10.

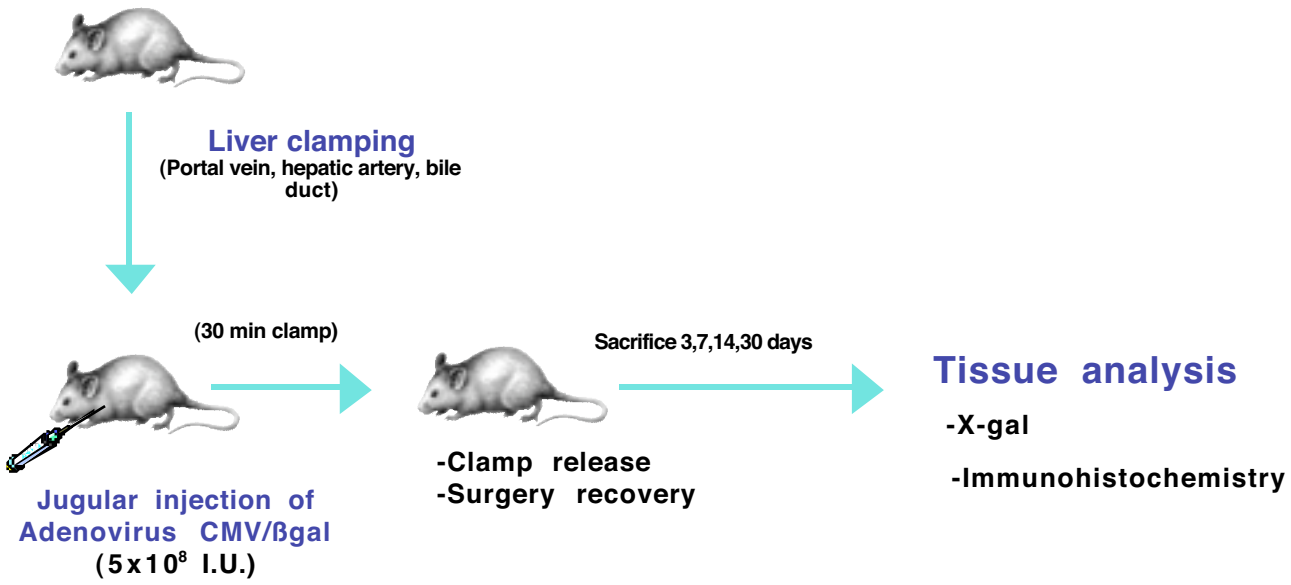


Figure 10. Scheme of experimental design used for adenovirus administration and sample analysis in mice.

1.1.2 Quantification of adenovirus in the bloodstream with and without portal clamp.

The main objective of the portal clamp was to maintain high concentrations of adenovirus in the circulation that may allow these vectors to transduce extra-hepatic tissues. Here we measured the clearance of Ad vectors in the bloodstream after jugular injection in mice with or without portal clamp. To this end, we took blood samples 5, 30, 35 and 60 minutes after vector injection (AdCMV/ß-gal $5x10^8$ IU) and we used 1µl of serum to infect HEK293 cells. Since the vector used in this experiment expressed the ß-gal gene under the ubiquitous CMV promoter, the ß-galactosidase activity in the infected cells could be quantitatively measured by a luminometric assay. As expected, when Ad were administered without portal clamp they were rapidly eliminated, and 30 min after the injection, the ß-galactosidase activity was dramatically reduced (Fig. 11). In contrast, in clamped animals the concentration of Ad in the circulation 30 min after

injection was similar to the concentration at 5 min, indicating that the portal clamp had prevented the liver-uptake of the vector during the clamp time. However, after opening the clamp, the virus was eliminated from circulation very rapidly, suggesting that the liver was able to remove the Ad immediately after the clamp release.

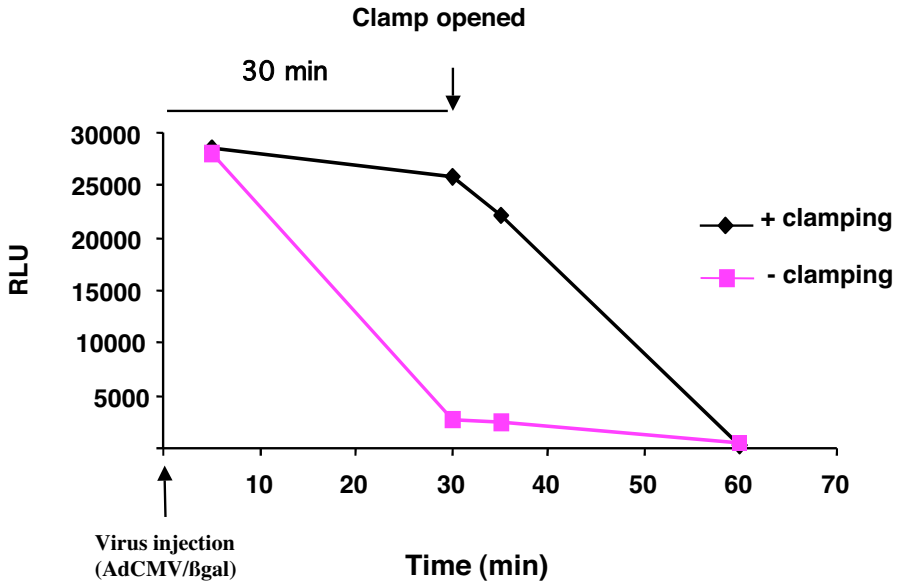


Figure 11. Clearance of Ad in bloodstream in mice with (+clamp) **or without** (-clamp) **portal clamp.** Serum samples were take 5, 30, 35 and 60 min after AdCMV/ß-gal vector injection into the jugular vein. HEK293 cells were infected with these serum samples and production of ß-galactosidase protein was measured by luminometry. The relative light units (RLU) value obtained by the luminometric assay is proportional to adenovirus concentration. The values of one representative mouse per group is shown.

1.1.3. Gene transfer to the pancreas in vivo.

Here we examined pancreas gene delivery in portal-clamped mice injected in the jugular vein with $5x10^8$ IU/mouse adenovirus particles carrying the ß-galactosidase marker gene (AdCMV/ß-gal). As expected, three days after viral injection we did not detect pancreas transduction in non-clamped animals (Fig. 12A,D). In contrast, when the hepatic circulation was closed for 30 min, pancreas gene transfer was achieved, as evidenced by ß-gal expression throughout the pancreas in an *in toto* analysis (Fig. 12B). Transduced pancreas showed groups of ß-gal expressing cells, which were consistent with islets of Langherhans (Fig. 12C, E, F). Lower doses of adenovirus ($5x10^6$ and $5x10^7$ IU/mouse) and shorter clamp duration (5 and 15 min) led to inefficient pancreas transduction. Furthermore, it has been reported that portal clamp longer than 30 min

produced severe liver ischaemia and higher viral doses induced hepatic damage (Ye *et al.*, 2000).

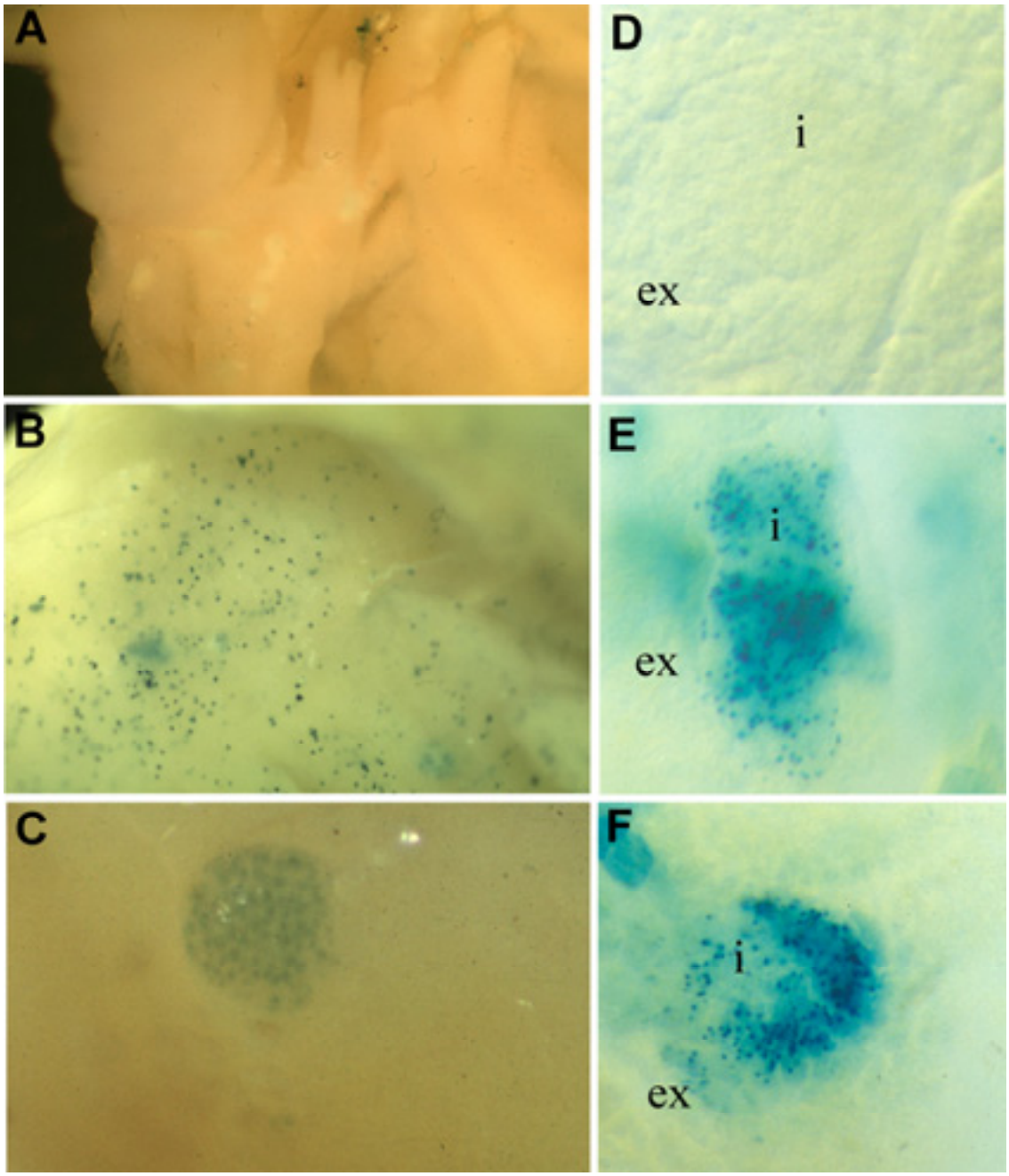


Figure 12. Pancreas gene transfer bypassing hepatic circulation. X-Gal staining of pancreas from non-clamped (**A** and **D**) and clamped (**B**,**C**,**E,F**) mice 3 days after adenovirus injection. (**A**) *In toto* analysis of pancreas showed no ß-gal expression in non-clamped animals. (**B**) Pancreas gene transfer was achieved in clamped mice. (**C**-**F**) Islets can be identified in the pancreatic parenchyma. Islets were not transduced in non-clamped animals (**D**), whereas large number of islet cells expressing ß-gal were detected in clamped mice (**C**,**E,F**). i, islets of Langerhans; ex, exocrine pancreas. Original magnification (**A**) ×25, (**B**) ×35, (**C**-**F**) ×128. Representative images from non clamped (n=5) and clamped mice (n=8).

1.1.4. Characterization of pancreatic transduction.

1.1.4.1. Transduction of endocrine pancreas.

Islet transduction was confirmed by specific ß-gal immunostaining of histological sections. Seven days after virus administration immunohistochemical analysis of pancreatic sections showed no ß-gal positive cells in islets of animals without portal clamp (Fig. 13A). In contrast, abundant ß-gal positive cells were observed within the islets of clamped animals (Fig. 13B,C). Distribution of transduced cells was within the core of the islet, where the insulin-producing cells reside, which agrees with the reported ß-cell tropism of adenoviruses (Sigalla *et al.*, 1997; Leibowitz *et al.*, 1999). Transduction of insulin-producing cells was confirmed by double immunostaining for insulin and ß-gal (Fig. 13D-F). ß-gal positive nuclei (green) were surrounded by cytoplasmatic insulin (red) (Fig. 13F), demonstrating that ß-cells were efficiently transduced. Similar results were obtained when adenoviral vectors carrying GFP (AdCMV/GFP) were injected. Double immunostaining for insulin and GFP showed ß-cell colocalization **(**Fig. 13G-I**).**

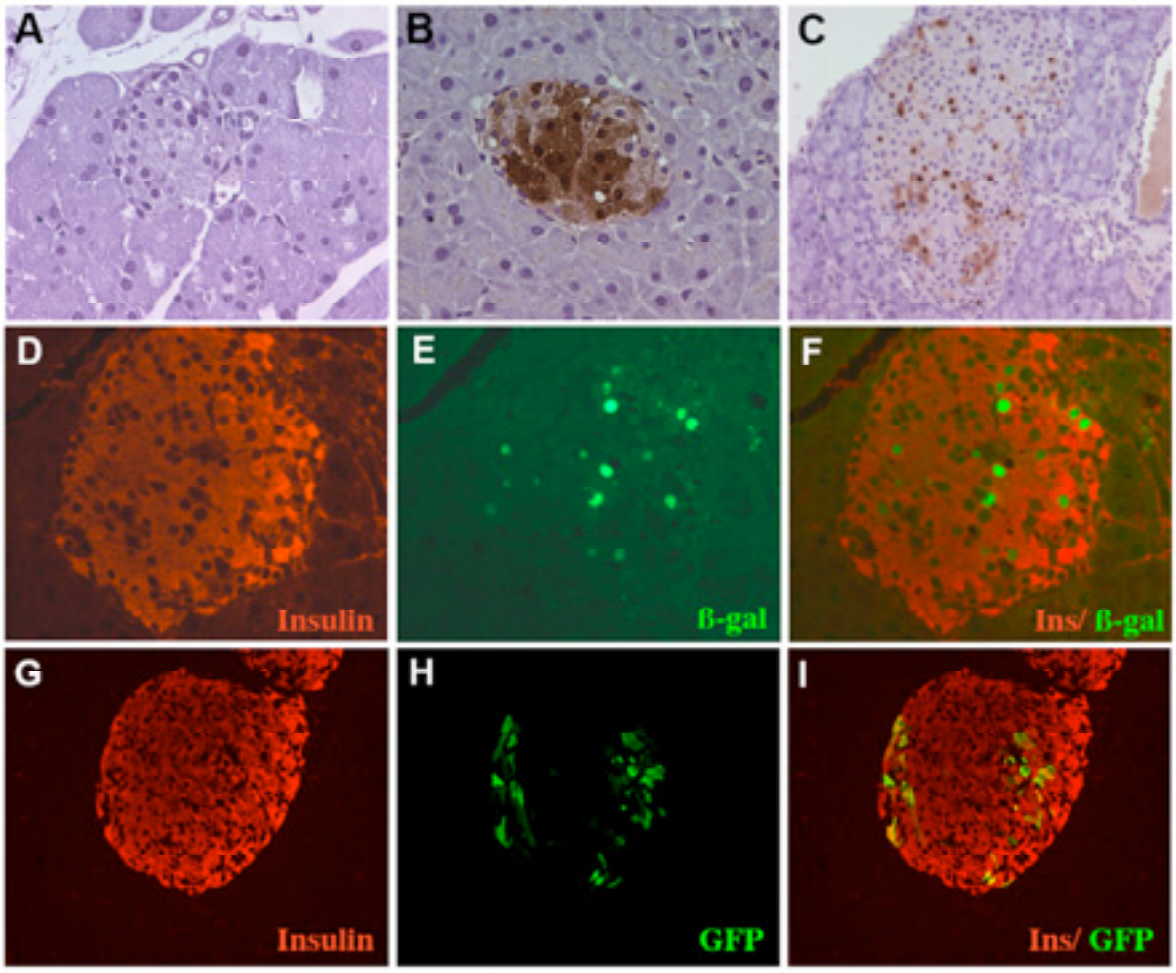


Figure 13. Pancreatic islets were efficiently transduced. (A-C) ß-gal immunohistochemical detection in pancreatic sections seven days after adenovirus administration. **(A)** No ß-gal positive nuclei were detected in the islets of animals without portal clamp. (**B** and **C**) ß-gal positive cells were observed within the islets of clamped animals. **(D-F)** Insulin (**D**) and ß-gal (**E**) double immunostaining was carried out in transduced islets. In the merged image ß-gal positive nuclei were clearly surrounded by cytoplasmatic insulin (**F**). (**G-I**) Insulin (**G**) and GFP (**H**) double immunostaining in transduced islets. In the merged image the yellow color documents colocalization (**I**). Original magnification **(A,B,D-F)** ×400 and **(C,G-I)** ×200**.**

Morphometric analysis was performed to determine the percentage of transduced islets per pancreas 7, 14 and 30 days after adenovirus injection. This analysis showed a high level of transduction since 71% of islets expressed ß-gal after 7 days (Fig. 14A). At day 14, about 40% of the islets were ß-gal positive. However, at day 30 a strong decrease in the number of ß-gal expressing islets was observed, with only 2 out of 5 animals presenting transduced islets (Fig. 14A). In addition, the

percentage of ß-gal-expressing cells per islet was measured. Seven days after vector delivery, about 20% of islet cells were positive for ß-gal (Fig. 14B). However, transduction within the islets also decreased significantly, with only about 5 and 4% of the ß-cells in the positive islets observed to be expressing ß-gal 14 and 30 days after vector administration, respectively (Fig. 14B).

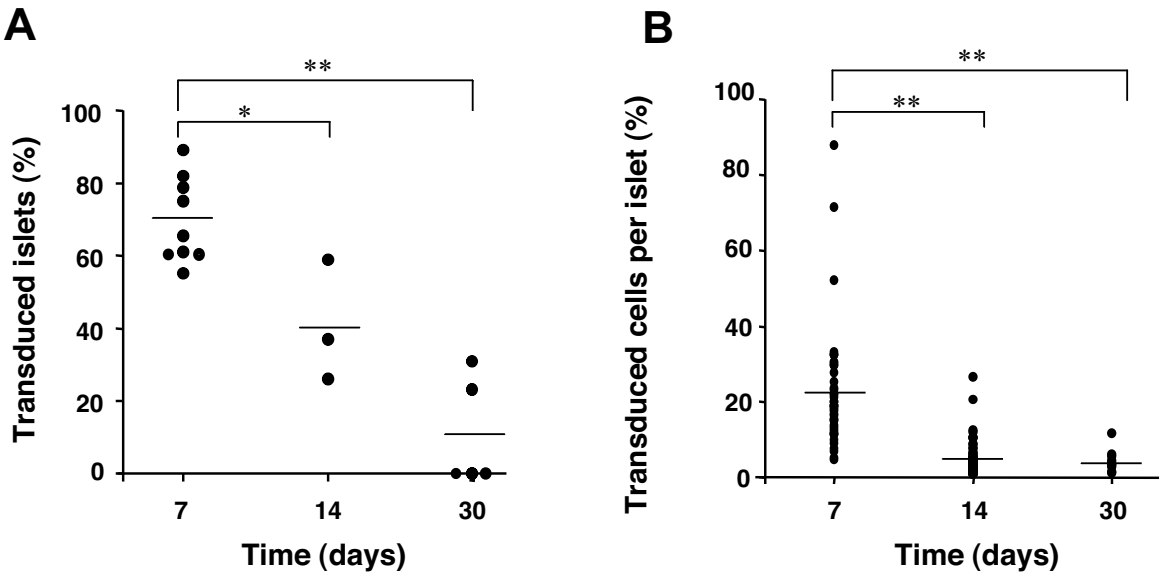


Figure 14. **Quantification of transduction**. **(A)** Percentage of transduced islets per mouse pancreas 7, 14 and 30 days after virus administration was determined as indicated in Materials and Methods. Each point represents an animal and the bar is the mean value. **(B)** Percentage of transduced ß-cells per islet 7, 14 and 30 days after virus administration was determined as indicated in Materials and Methods. All transduced islets from 3 animals were analyzed at day 7, 3 animals at day 14, and from 2 animals at day 30. Each point represents an islet and the bar is the mean value. * $p<0.05$, ** $p<0.01$

To evaluate the role of the immune response in the loss of ß-gal-expressing cells, we measured the incidence and severity of insulitis in Ad treated mice. We found that only a small percentage (less than 20%) of ß-gal-expressing islets presented perinsulitis, which did not progress to severe insulitis one month after Ad injection (Fig. 15A,B). Furthermore, mice remained normoglycemic one month after viral injection (non-clamped mice, 130±8 mg/dl *vs*. clamped mice, 122±9 mg/dl). This indicated that pancreatic ß-cell function was not altered.

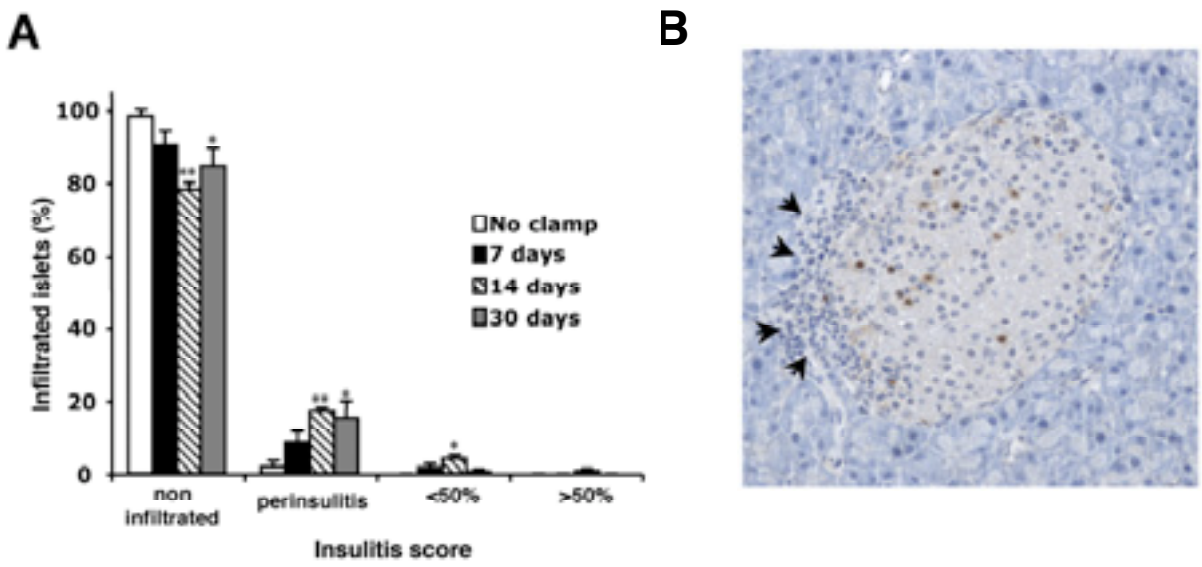


Figure 15. Lymphocytic infiltration of islets. (**A**) Islets were scored for inflammation as described in Material and Methods. Histograms depicts percentage of non infiltrated islets, perinsulitis, moderate insulitis (<50% of the islet infiltrated) and severe insulitis (>50% of the islet infiltrated). Lymphocytic infiltration was measured 7 (n=8), 14 (n=3), and 30 days (n=3) after the adenovirus injection in clamped mice, whereas non clamped mice (No clamp, n=3) were used as controls. (**B**) Representative image of perinsulitis in a ß-gal expressing islet is shown. ß-gal positive nuclei are brown. Arrows point to lymphocytes. Original magnification 400X

1.1.4.2. Transduction of exocrine pancreas.

In addition to ß-cells, ß-gal expression was also noted in exocrine pancreas (Fig. 16). However, few acinar cells were X-Gal stained, though their distribution was homogeneous over the entire pancreas (Fig. 16).

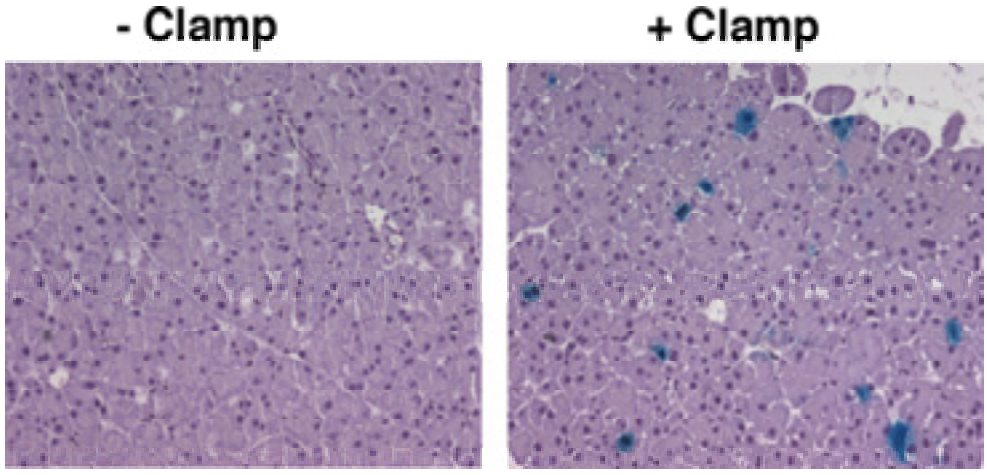


Figure 16. Acinar cell tansduction. After *in toto* X-Gal staining, pancreas was paraffin embedded, sectioned and counterstained with hematoxylin. (-clamp) Exocrine pancreas was not transduced. (+clamp) Scattered acinar cells were transduced in the pancreas of clamped animals. Original magnification 200X

Seven days after adenovirus administration, the percentage of acinar cell transduction was about 0.3% of total exocrine pancreatic cells (Fig. 17). No significant decrease in the number of ß-gal-expressing acinar cells was observed at day 30 (Fig. 17**)** which was consistent with the absence of lymphocytic infiltration (Fig. 18A).

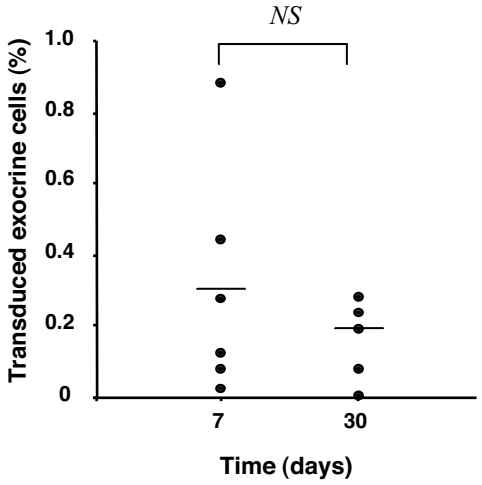


Figure 17. Quantification of exocrine pancreas transduction. Percentage of transduced exocrine cells 7 and 30 days after virus administration was determined as indicated in Material and Methods. Each point represents an animal and the bar is the mean value. *NS* Not significant (p=0.45).

When adenoviruses ($5x10^8$ IU) was directly injected into the pancreas, increased exocrine cell transduction was observed around the area of injection (Fig. 18A). However, severe infiltration of inflammatory cells was also noted (Fig. 18B), which led to loss of ß-gal expressing cells and most likely compromised organ functionality. This agrees with published results which show that between days 3-7 after adenovirus injection in immunocompetent mice, the expression of the foreign genes drops strongly (McClane *et al.*, 1997a). Thus, although the level of transduction of exocrine pancreas is lower, systemic delivery in portal clamped animals is advantageous since the persistence of the engineered acinar cells may allow long-term expression of foreign genes. Gene transfer to acinar cells may be of interest for producing proteins that may act in the

pancreas or be exported to the ductal lumen or bloodstream (Goldfine *et al.*, 1997; Vickers *et al.*, 1997; Schmid *et al.*, 1998).

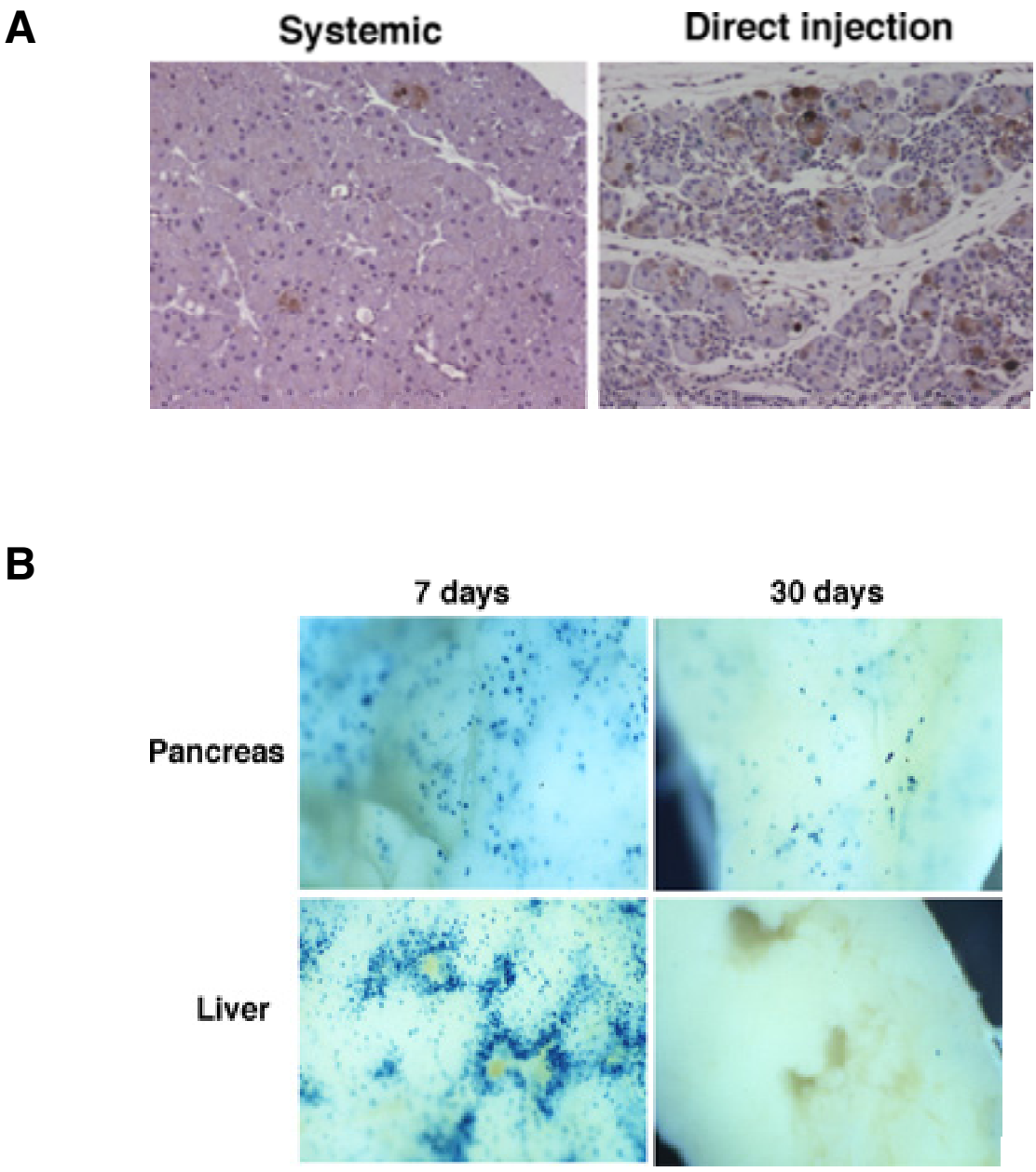


Figure 18. Persistence of ß-gal expression. (A) Lymphocytic infiltration in the exocrine pancreas 7 days after virus administration either by systemic delivery or by direct injection. ß-gal immunostaining (brown) in pancreatic sections demonstrates transduced cells. Direct injection of adenoviruses into the pancreas led to increased ß-gal expressing cells but severe inflammation was observed. Original magnification 200X**. (B)** *In toto* X-gal staining showed persistence of ß-gal in the pancreas, but not in the liver 30 days after vector administration. Original magnification; pancreas ×25 and liver ×35.

1.1.5. Specific expression of the gene of interest in beta cells using the rat insulin promoter.

Gene transfer to murine pancreas *in vivo* can be achieved by systemic delivery of Ad vectors after clamping hepatic circulation. However, systemic administration of Ad vectors may result in transduction of other organs such as liver. To prevent expression of the gene of interest in undesired tissues, cell-type specific promoters can be used to direct the expression to ß-cells or acinar cells. Thus, we generated Ad vectors carrying the ß-galactosidase (ß-gal) marker gene under the control of rat insulin-II promoter (RIP-II) and we injected these vectors in mice using the portal clamp technique.

1.1.5.1. Generation of adenoviral vectors carrying RIP-II/ß-gal.

To demonstrate the selective expression of the gene of interest in ß-cell we generated first generation adenoviral vectors (FG-Ad) containing ß-galactosidase as a marker gene under the control of rat insulin promoter-II. The system used in this study to generate these vectors relied on standard cloning in bacteria and subsequent transfection of recombinant chromosomes into mammalian cells for virus production (see Materials and Methods section 4.6 and 5). Two plasmids were required to generate the adenoviral genome with the expression cassette. The first shuttle plasmid contains two arms for recombination flanking the expression cassette (PTG6600), and the second plasmid contains the E1 deleted adenoviral genome (pKP1.4Δ). The insertion of the expression cassette into the Ad genome is carried out by recombination of the two linearized plasmid using a specific bacterial background (BJ 5138) with high

recombination efficiency. The steps to generate the Ad vector are summarized in Fig. 19 and Fig. 20. First, the CMV promoter of the PTG6600 plasmid was removed by digestion with *BglII* and *HindIII*, and was substituted with the RIP-II promoter released from Psp72 RIP-II plasmid using *BamHI* and *HindIII* restriction sites. Since *BamHI* and *BglII* sites generated compatible ends after digestion, ligation of the two fragments with T4 ligase resulted in directional cloning of the RIP-II promoter in the PTG6600 plasmid (PTG6600 RIP-II). The next step was the cloning of the ß-galactosidase gene into the PTG6600 RIP-II. The ß-gal gene was removed from the pCMVß plasmid by digestion with *NotI* and was subcloned into the *NotI* site of the PTG6600 RIP-II multicloning site (MCS) by standard ligation. Thus, the shuttle plasmid containing the RIP-II/ß-gal cassette flanked by Ad5 genome regions was obtained (PTG6600 Rip-II/ß-gal) and was linearized with the *BstEII* restriction enzyme for the recombination. The plasmid containing the E1 deleted genome (pKP1.4Δ) was also linearized using the *SwaI* enzyme. Both linearized plasmids were co-transformed in BJ bacteria and were grown in the presence of ampicilin. Only plasmids with circularized form should be able to generate colonies in these conditions. These colonies were cultured in 2 ml of LB medium with ampicillin, and plasmid DNA was extracted from the bacteria (minipreps). This DNA was then transformed into another bacterial background (*Xl2-blue*) to avoid further recombination of the Ad genome. Then, after plasmid DNA isolation from these bacteria, the Ad genome containing the expression cassette was checked using several restriction digestions. The correct plasmid (pKP1.4 RIP-II/ß-gal) digested with *PacI* was used to transfect HEK293 cells in order to generate the viral particles. The production method of the adenoviral vectors is described in Material and Methods

(section 5). The titer of the preparation used in these experiments was $6.75x10^{11}$ pp/ml and $5x10^{10}$ IU/ml.

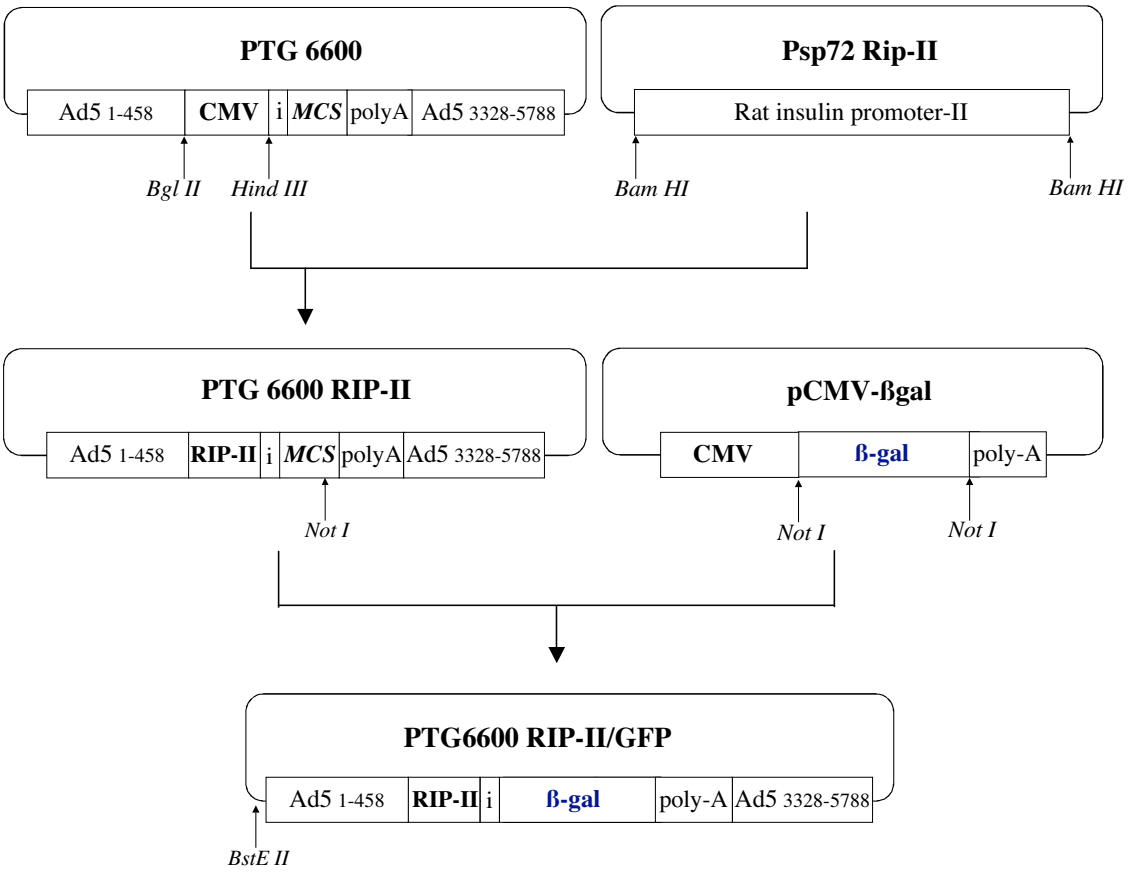


Figure 19. Construction of the shuttle plasmid PTG6600 RIP-II/ß-gal, which contains the expression cassette RIP-II/ß-gal flanked by two arms of Ad5 DNA regions for recombination with the E1 deleted Ad5 genome. MCS=multicloning site, i=ß-globin intron, pA=polyA=polyadenylation signal.

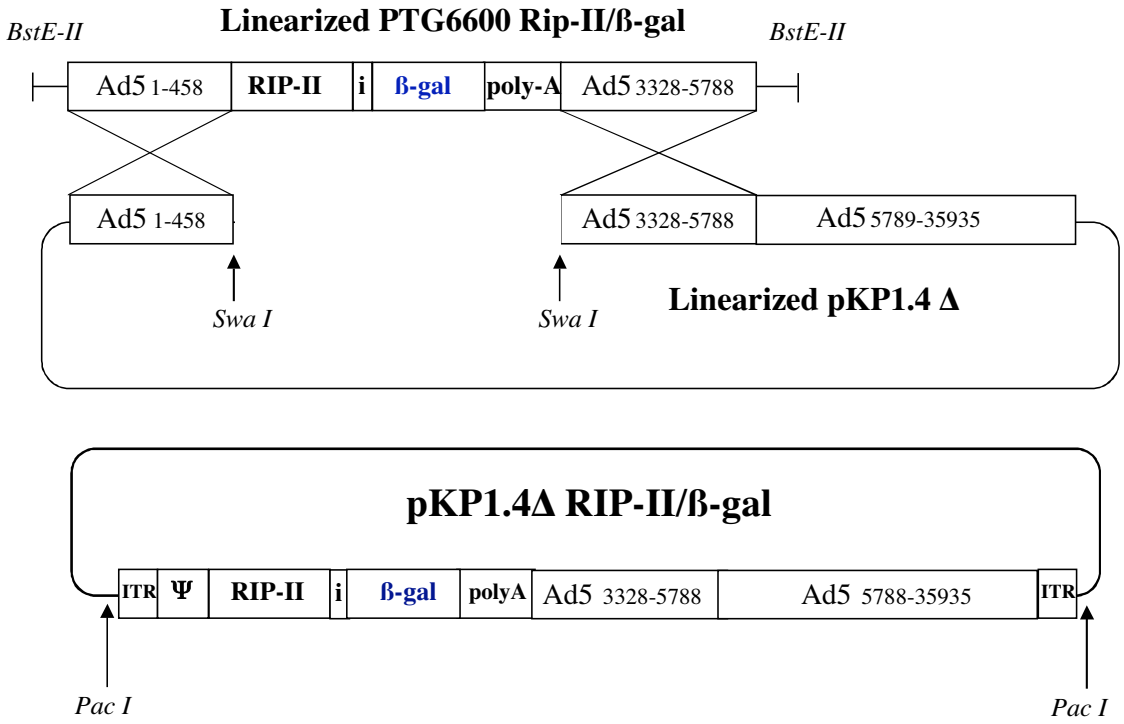


Figure 20. Construction of the E1 deleted adenoviral genome carrying the RIP-II/ß-gal expression cassette (pKP1.4Δ RIP-II/ß-gal) by homologous recombination. The shuttle plasmid PTG6600 Rip-II/ß-gal linearized with *BstEII* was co-transformed in BJ bacteria together with the pKP1.4Δ plasmid linearized with *SwaI*. The recombination event is shown, and the resultant plasmid was named pKP1.4Δ RIP-II/ß-gal. ITR=inverted terminal repeat, i=ß-globin intron, polyA=polyadenylation signal, Ψ=packaging signal.

1.1.5.2. In vivo administration of adenoviral vectors expressing ß-gal under the control of the rat insulin promoter.

Adenoviral vectors expressing ß-gal under the control of the rat insulin promoter-II were injected into the bloodstream of mice with clamped hepatic circulation (10^9 IU/mice) using the portal clamp procedure described in the previous experiments. Immunohistochemical detection of the marker gene revealed that ß-gal expression occurred specifically in ß-cells, and no expression was found in liver or exocrine pancreas (Fig. 21).

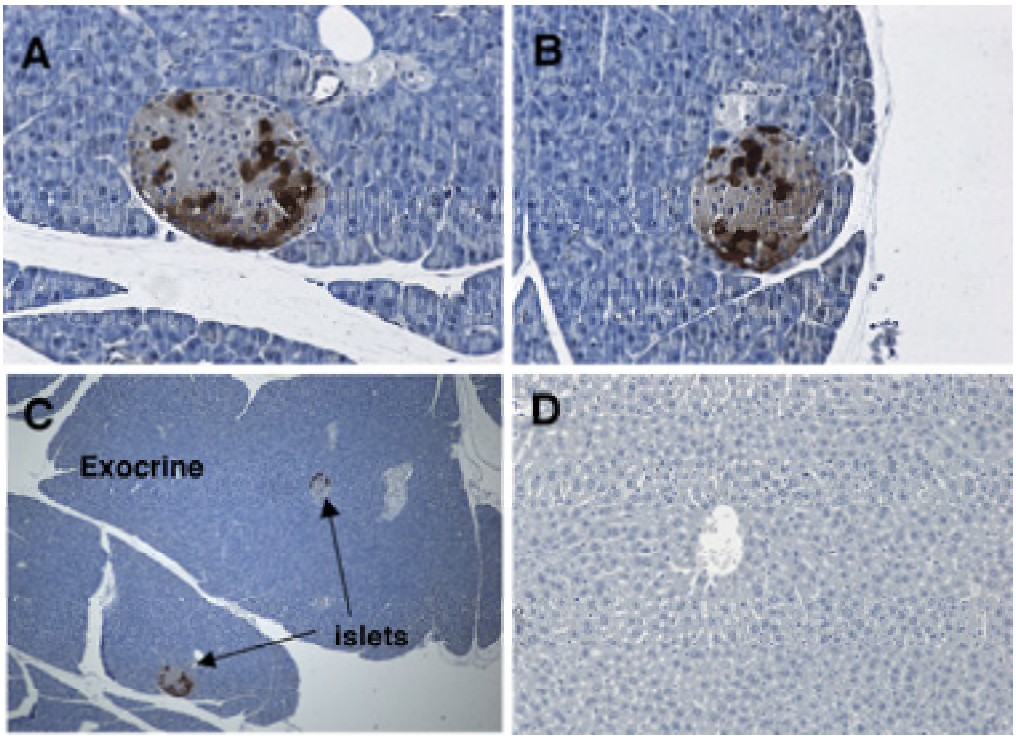


Fig. 21. Expression of the ß-gal gene specifically in ß-cells using adenoviral vectors carrying the rat insulin promoter-II/ß-gal expression cassette gene. After immunohistochemical detection of ß-gal (brown) significant expression was observed in pancreatic islets (B-D). In contrast, no expression was detected in the exocrine pancreas (B-D) or the liver (D). Original magnification; x400 (A), x200 (B), x40 (C), x100 (D).

1.2. Gene transfer to murine pancreas *in vivo* using helper-dependent adenoviral vectors.

Adenoviral gene transfer to pancreatic islets using the clamp technique has raised new expectations for pancreatic gene therapy. However, FG-Ad vectors showed high toxicity and short duration of the transgene expression due to immune response against the transduced cells (Liu and Muruve, 2003). It is well known that even in the absence of the E1 region in the FG-Ad genome, expression of other viral genes occurred at low levels and this expression was enough to trigger the immune response (Yang *et al.*, 1994a). To avoid the adverse effects of FG-Ad vectors, helper-dependent adenoviral (HD-Ad) vectors can be used. These vectors are deleted of all viral coding sequences. HD-Ad retain the advantages of FG-Ad, including high-efficiency of *in vivo* transduction and high-level of transgene expression. However, owing to the absence of viral gene expression in transduced cells, these HDAds are able to mediate high-level, long-term transgene expression in the absence of chronic toxicity. However, no data regarding pancreas transduction with these vectors have been reported so far. Since HD-Ad have the same capsid of FG-Ad, the tropism of the vector should be the same when equal doses are used. Here we investigated whether gene transfer to ß-cells can be achieved by systemic injection of HD-Ad in mice using the portal clamp tecnique.

1.2.1. Production of helper-dependent adenoviral vectors carrying marker genes.

To generate HD-Ad vectors, three components are required. Firstly, the plasmid which contains the expression cassette, ITRs, packaging signal and stuffer DNA. Secondly, the helper virus that is an E1 deleted FG-Ad vector with the packaging signal flanked by *loxP* sites; and lastly, a human cell line that expresses the E1 protein and *Cre* recombinase. The plasmids used for generation of HD-Ad vectors are shown in Fig. 22. The pGS46 plasmid carried the CMV/ß-gal expression cassette while the pFK7 carried the CMV/GFP expression cassette. In addition to the expression cassettes, the pGS46 and pFK7 vectors contained the ITRs, packaging signal and stuffer DNA from HPRT (Edwards *et al.*, 1990) and C346 human loci (Andersson *et al.*, 1995). The *PmeI* site is used to remove bacterial sequence to liberate the ITRs and generate a linear Ad genome to be used in the transfection. The helper virus (HV) used to propagate the HD-Ad genome was the GS102. This vector has the Ad5 ITRs, Ad5 sequences from 3523 bp to 35935 bp, the packaging signal flanked by *loxP* sequences, and a insertion of 4.6 kb from λ DNA. The insertion of an extra 4.6 Kb DNA was used to avoid packaging of replication-competent virus generated by recombination between HV and the E1 region of the producer cell line, since this potential recombination would result in Ad genomes >39 kb. In addition, the HV genome is usually larger than the HD-Ad, and this can help to separate the HD-Ad vectors from HV after CsCl gradient ultracentrifugation. The cell line used in these experiments was the CRE66 that was derived from HEK293 cells and expresses the Cre recombinase. The production protocol is described in Material and Methods (section 6).

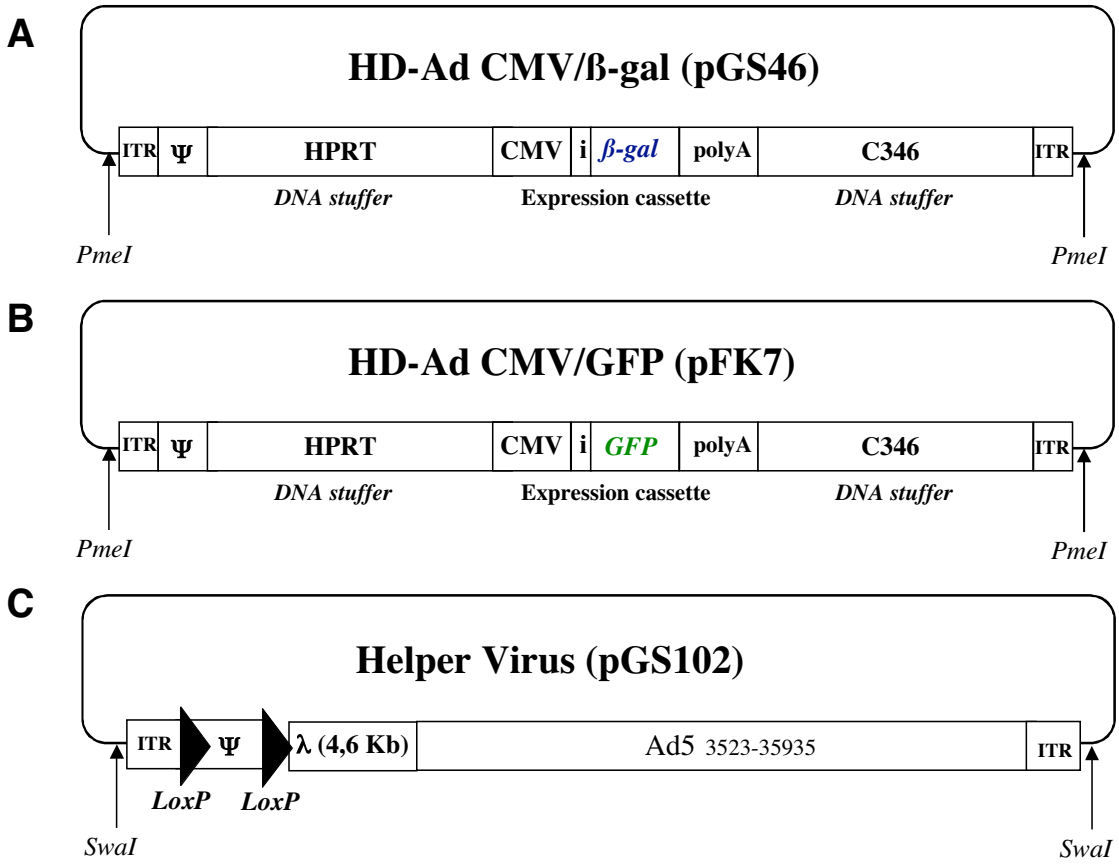


Figure 22. Schematic representation of helper-dependent adenoviral constructs carrying the ß-galactosidase (**A**) or GFP (**B**) gene under the control of the CMV promoter. The helper virus used to propagate the HD-Ads genomes in these studies is shown in (**C**). The *PmeI* site in the HD-Ads and the *SwaI* site in helper virus are restriction sites used to remove the bacterial sequences prior transfection, to generate the viral particles. ITR=inverted terminal repeats, i=intron, polyA=polyadenilation signal, Ψ=packaging signal.

1.2.2. In vivo administration of helper-dependent adenoviral vectors carrying marker genes.

The titer of the viral preparations used for *in vivo* studies were $4.5x10^{11}$pp/ml for HD-Ad CMV/GFP and $1x10^{12}$ pp/ml for HD-Ad CMV/ß-gal. Two hundred microliters of virus stock was injected into the jugular vein in mice undergoing portal clamp. Mice were sacrificed 7 days after the injection and both liver and pancreas were removed, fixed, and embedded in paraffin. After immunohistochemical analysis,

expression of both GFP (Fig 23A,B) and ß-gal (Fig 23C,D) marker genes were detected in pancreatic islets. However, prominent GFP expression was also detected in the liver of these mice (Fig 24), because the expression of the marker gene was driven by the ubiquous CMV promoter. As demonstrated with FG-Ad, the gene of interest can be selectively expressed in the ß-cells using the rat insulin promoter.

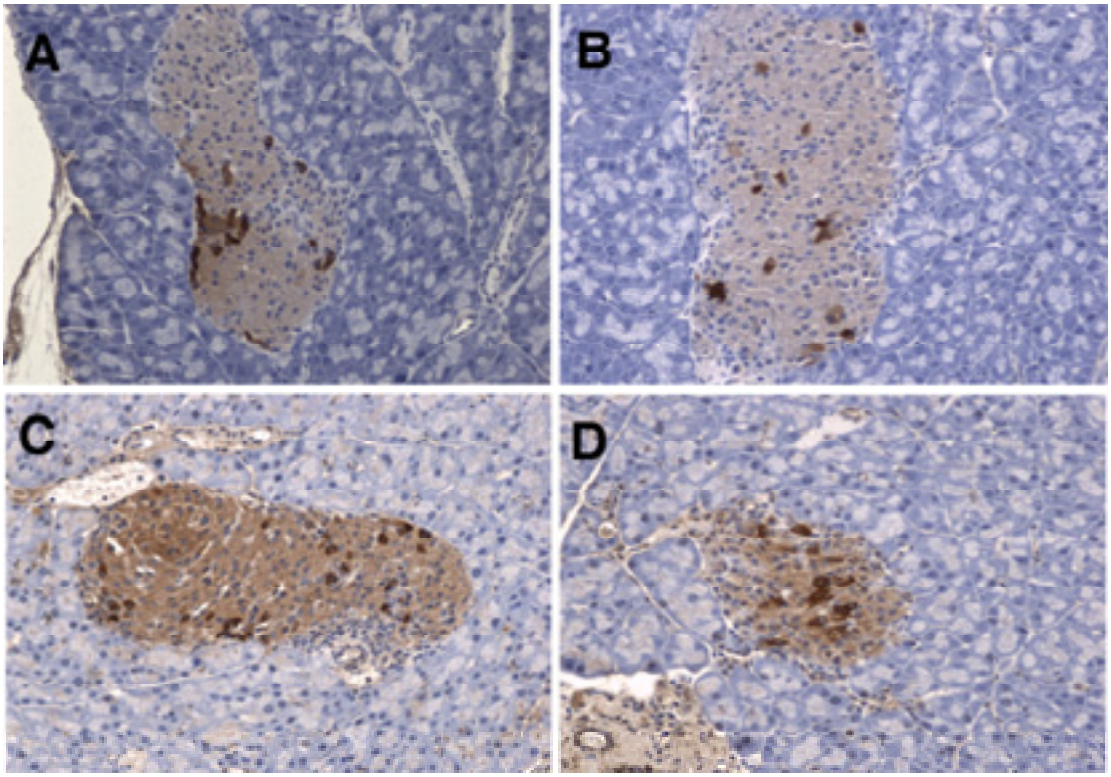


Figure 23. Expression of the marker gene in pancreatic islets. The upper panel (**A,B**) showed islets transduced with HD-Ad CMV/GFP (injection of $8x10^{10}$pp/mouse) and the lower panel (**C,D**) showed islets transduced with HD-Ad CMV/ß-gal (injection of $2x10^{11}$pp/ml). Original magnification x200.

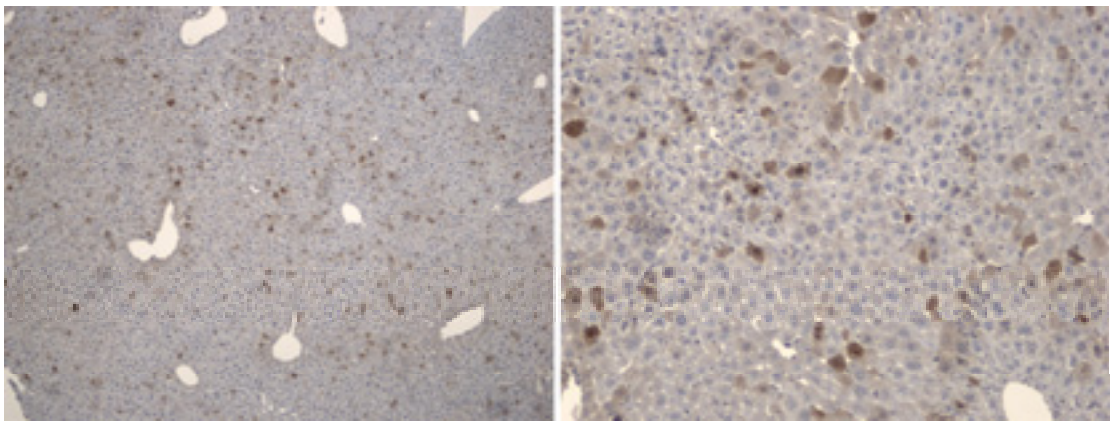


Figure 24. Expression of the marker gene in the liver. High expression of GFP in the liver was observed after injection of HD-Ad CMV/GFP ($8x10^{10}$pp/mouse) into the jugular vein in mice with portal circulation clamped. Original magnification; x40 (left) and x200 (right).

1.3. Gene transfer to murine pancreas in vivo using adeno-associated viral vectors.

Adeno-associated viral vectors (AAV) are promising tools for gene transfer to pancreas since these vectors have low immunogenicity and are derived from non-pathogenic viruses. Among all serotypes the AAV8 seems to be the most appropriate for pancreatic transduction in mice (Wang *et al.*, 2004a; Loiler *et al.*, 2005; Wang *et al.*, 2006).

1.3.1. Production of AAV vectors carrying marker genes.

To examine the ability of AAV vectors to transduce the pancreas, we have generated AAV8 vectors carrying marker genes under the control of both ubiquitous (CMV, CAG) and ß-cell-specific (RIP-II) promoters. The AAV CAG-GFP-WPRE vector was previously generated in our laboratory. The expression cassette was composed of the chicken ß-actin promoter, including the CMV enhancer (CAG), the eGFP cDNA, and the woodchuck post-transcriptional regulatory element (WPRE). The WPRE has been shown to increase RNA stability and transgene expression (Mian et al. 2004). To generate the AAV vector expressing the GFP marker gene under the control of rat insuilin promoter-II, the eGFP cDNA was removed from peGFPc1 plasmid using the *NheI* and *Asp718I* restriction sites and was inserted into the PTG6600 RIP-II plasmid using the same sites, by standard ligation. The RIP-II/GFP expression cassette was subsequently excised using *SgrAI* and *MfeI* restriction sites and was blunt-ended using the DNA polymerase I fragment (Klenow). In the AAV genome, the CMV standard cassette of the AAV-MCS vector was eliminated by *NotI* digestion and the

AVV plasmid was also blunt-ended using the Klenow fragment. The ligation of blunted RIP-II/GFP and AAV plasmid was carried out by standard ligation with T4 ligase and chemically competent cells (*XL10-gold*) were transformed. The resultant vector was named pAAV-RIP-II/GFP. A schematic representation of the cloning procedure is shown in Fig. 25. Production methods for the generation of recombinant AAV vectors are described in Material and Methods (section 7).

The titer of the vectors used in these studies was: $5x10^{11}$ vg/ml for AAV8 CMV/ßgal, $1.5x10^{11}$ vg/ml for AAV8 CAG-GFP-WPRE, and $7x10^{11}$ vg/ml for AAV8 RIP-II/GFP.

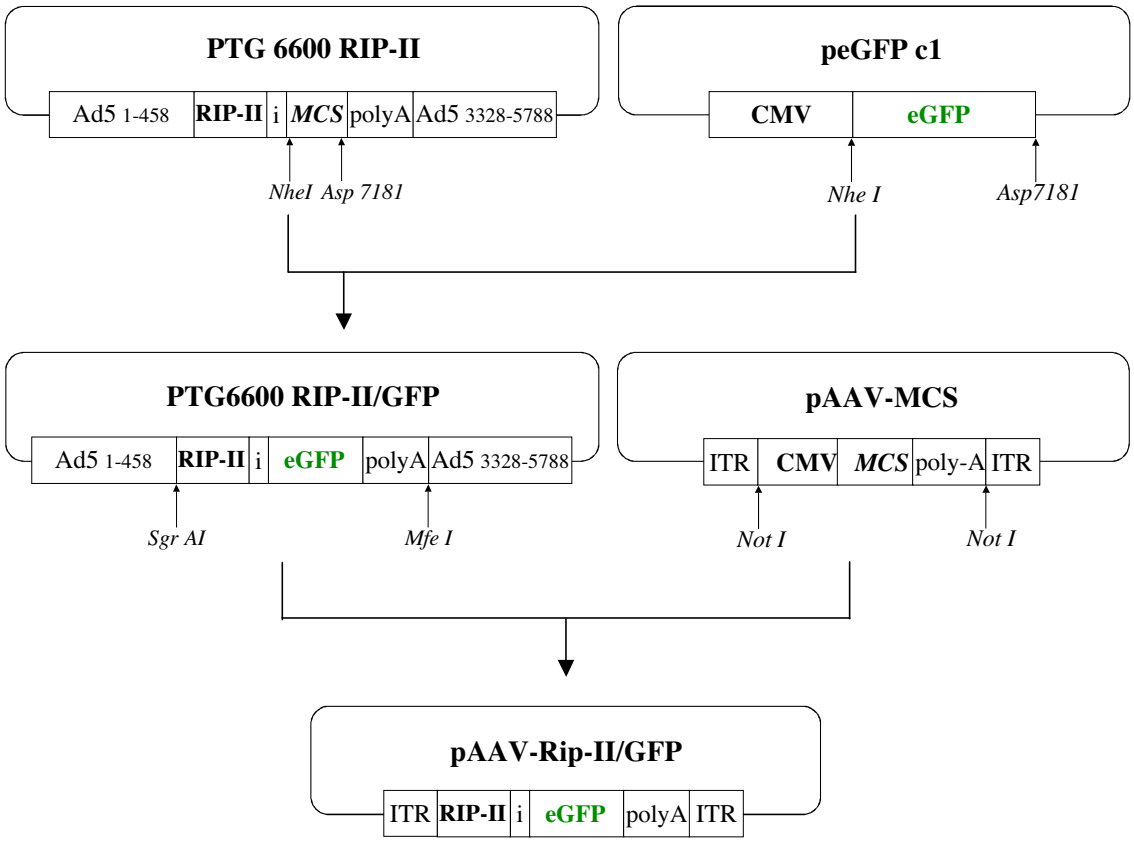


Figure 25. Schematic representation of the cloning steps followed to generate the AAV genome with the RipII/GFP expression cassette. ITR=inverted terminal repeats, i=intron, polyA=polyadenilation signal, Ψ=packaging signal, MCS=Multi Cloning Site.

1.3.2. Comparison of different routes of administration for pancreatic gene transfer using AAV vectors.

1.3.2.1. Intravascular delivery.

In this study we investigated whether the AAV8 vectors possess a natural tropism for pancreatic tissue when administered intravenously. One report has shown that injection of $7x10^{11}$ vg/mouse into the tail vein resulted in pancreas transduction (Nakai *et al.*, 2005). However, when using such a high dose of AAV vector, all the studied tissues showed expression of the marker gene. Here we further investigated whether lower doses of AAV8 could transduce preferentially or not the pancreas after intravascular delivery. To this end, we inject 10^{10} vg/mouse of AAV8 CMV/ß-gal into the jugular vein and 30 days after ß-gal expression was analyzed in the liver and the pancreas. No expression of the marker gene was found (data not shown). Therefore, we decided to increase the viral dose and to reduce the time for the analysis to avoid the elimination of the transduced cells by an eventual immune response. Thus, we injected $5x10^{10}$ vg/mouse of the same vector and these mice were sacrificed 7 days later. Expression of the marker gene was found in the liver but not in the pancreas (Fig 26). These data suggested that AAV8 did not have a high tropism for pancreatic tissue when injected intravenously. Next, we investigated whether by bypassing the liver, these vectors might target the pancreas when injected systemically. To this end, we injected $5x10^{10}$ vg/mouse of AAV8 CMV/ß-gal into the jugular vein in mice with the hepatic circulation clamped for 30 min. Mice were sacrificed 7 days after, and expression of ß-gal was found in the liver but not in the pancreas. Together, these data suggest that although transduction of the pancreas is possible using very high doses of AAV8, this

vector did not have a strong tropism for this tissue when administered intravenously. In addition, liver uptake of the viral vectors was not the main explanation for the low transduction efficiency, since bypassing liver circulation did not result in significant increase of pancreatic transduction.

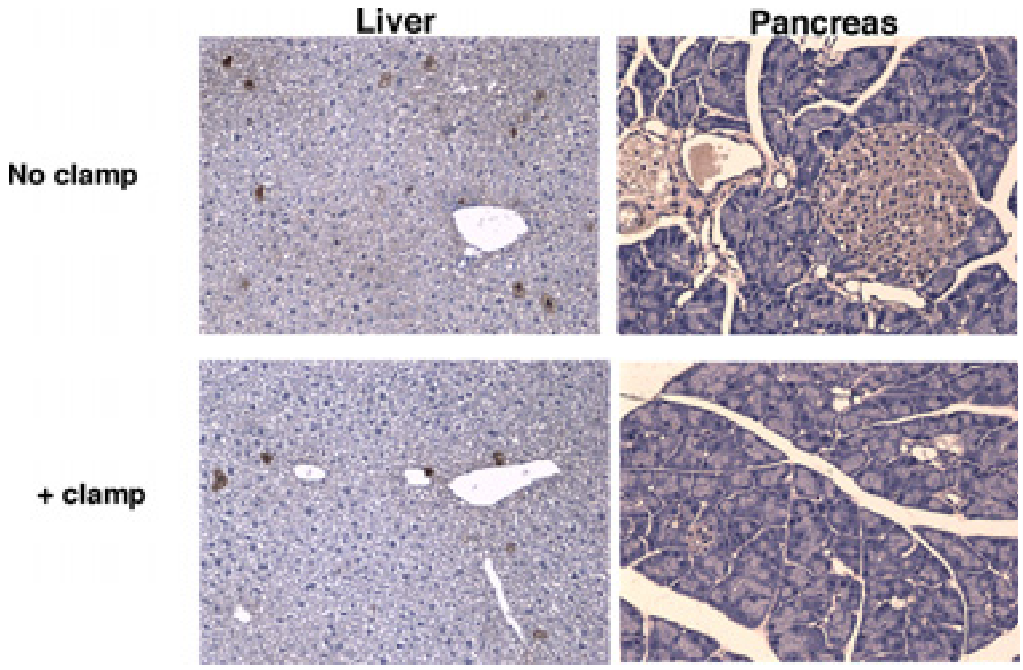


Figure 26. Analysis of liver and pancreas transduction after intra-jugular injection of AAV8 vectors in mice with or without portal clamp. ß-gal expression was found in the liver, but not in the pancreas 7 days after vector administration. The clamp of the hepatic circulation did not result in significant differences in transduction. Original magnification; x100 (liver), x200 (pancreas) .

1.3.2. 2. Intraperitoneal delivery.

Intraperitoneal delivery is a common route of administration in mice. The absorption from the peritoneum to the bloodstream of a certain compounds, such as glucose or insulin, can occur in the order of seconds. It has been recently shown that intraperitoneal injection of AAV8 vectors in neonatal mice resulted in wide distribution of the vector, with many tissues, including the pancreas, expressing the marker gene (Wang *et al.*, 2005). In this study, 1 day-old mice and a dose of $2x10^{11}$ vg/mouse of AAV8 vectors were used. Nevertheless, this dose is equivalent to 10^{14} vg/kg and such a high dose is unlikely to be used in humans. Here we further investigated whether intraperitoneal delivery could be an efficient method to transfer genes to the pancreas using lower doses of the vector. To this end, AAV8 CAG-GFP ($1.5x10^{10}$ vg/mouse) and AAV8 Rip-II/GFP ($7x10^{10}$ vg/mouse) vectors were intraperitoneally injected into 1 week-old mice. One week after, mice were sacrificed and GFP expression was analyzed. Prominent expression of the marker gene was found in the heart and the liver of mice injected with the CAG-GFP construct (Fig. 27), although no transduction was observed in the pancreas or islets (Fig. 28). In addition, no expression in any tissue, including the islets, was found using the selective rat insulin promoter-II (Fig. 27 and Fig. 28). Thus, intraperitoneal injection of AAV8 seems to be a very efficient delivery method to target the myocardial muscle or the liver but not the pancreas.

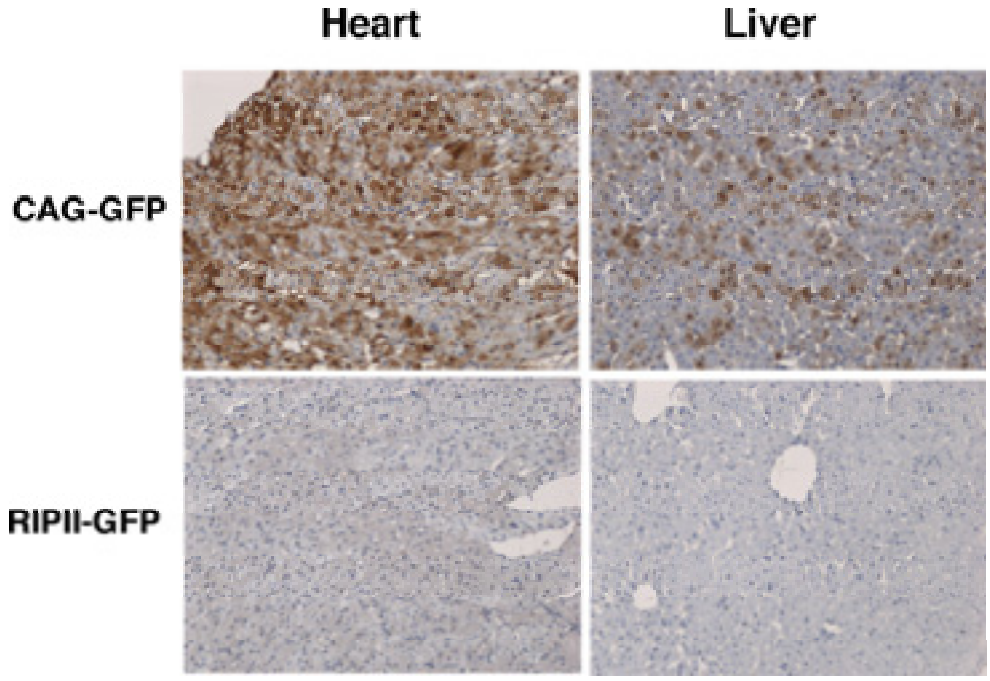


Figure 27. Gene transfer to the liver and the heart by intaperitoneal delivery of AAV8 vectors. Prominent expression of the marker gene was found in the heart and the liver of mice injected intraperitoneally with AAV8 CAG-GFP ($1.5x10^{10}$ vg/mouse). The use of the rat insulin promoter-II (AAV RIP-II/GFP $7x10^{10}$ vg/mouse) avoided expression of the marker gene in the heart and the liver. Original magnification x100.

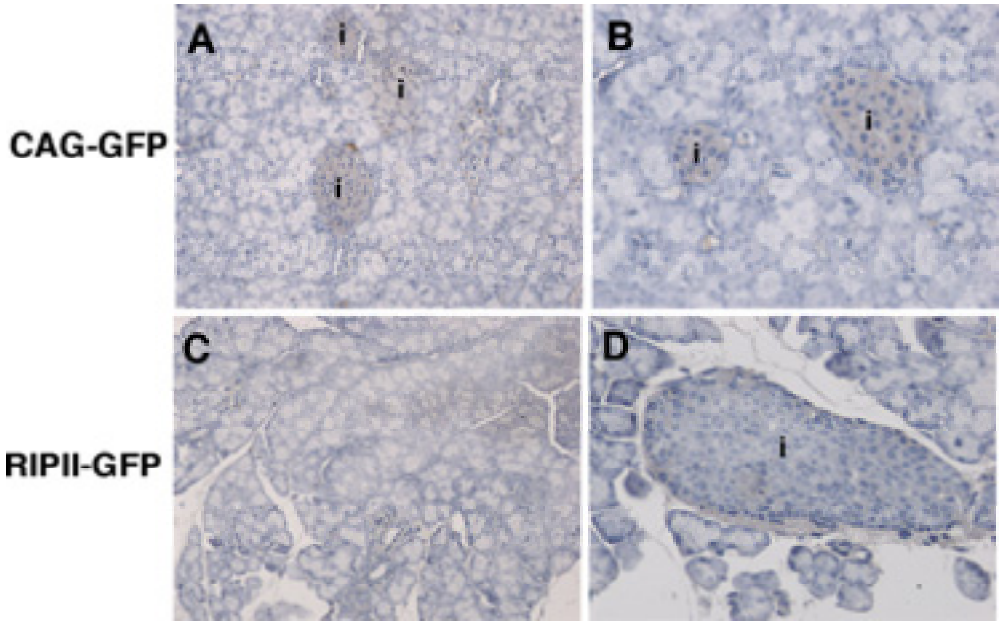


Figure 28. Gene transfer to the pancreas by intaperitoneal delivery of AAV8 vectors. No expression was detected in the pancreas and islets of mice injected either with CAG-GFP GFP ($1,5x10^{10}$ vg/mouse) (**A,B**) or RIP-II/GFP ($7x10^{10}$ vg/mouse) (**C,D**) intraperitoneally. Original magnification x100 (**C**), x200 (**A,D**), x200 (**B**). i=islet.

1.3.2.3. Intraductal delivery.

The exocrine pancreas consists of acinar cells that produce and secrete a variety of digestive enzymes, such as proteases, lipases and nucleases, and the ductal system, which transports the digestive enzymes and bicarbonate ions to the intestine, where they contribute to the digestion of food. The main pancreatic duct converges with the biliary duct before draining into the intestine through the sphinter of Oddi. Therefore, it is possible to carry out retrograde injection of viral vectors from the common bile duct to the pancreas. Indeed, a similar procedure called endoscopic retrograde cholangiopancreatography (ERCP) is used in humans for exploration/intervention of ductal pathologies. This route of administration has been used in mice and rats to transduce the pancreas using adenoviral vectors (Raper and DeMatteo, 1996; Taniguchi *et al.*, 2003; Kozawa *et al.*, 2005) and also more recently with AAV vectors (Loiler *et al.*, 2005). Among the several serotypes studied, AAV8 was the most efficient vector in mice, although quite high viral doses ($5x10^{11}$ vg/mouse) were required in these experiments (Loiler *et al.*, 2005). This technique was set up in our laboratory and similar doses to those used in intravenous and intraperitoneal experiments were administered (above; sections 1.3.2.1 and 1.2.2.3). A schematic representation of the procedure is shown in Fig. 29.

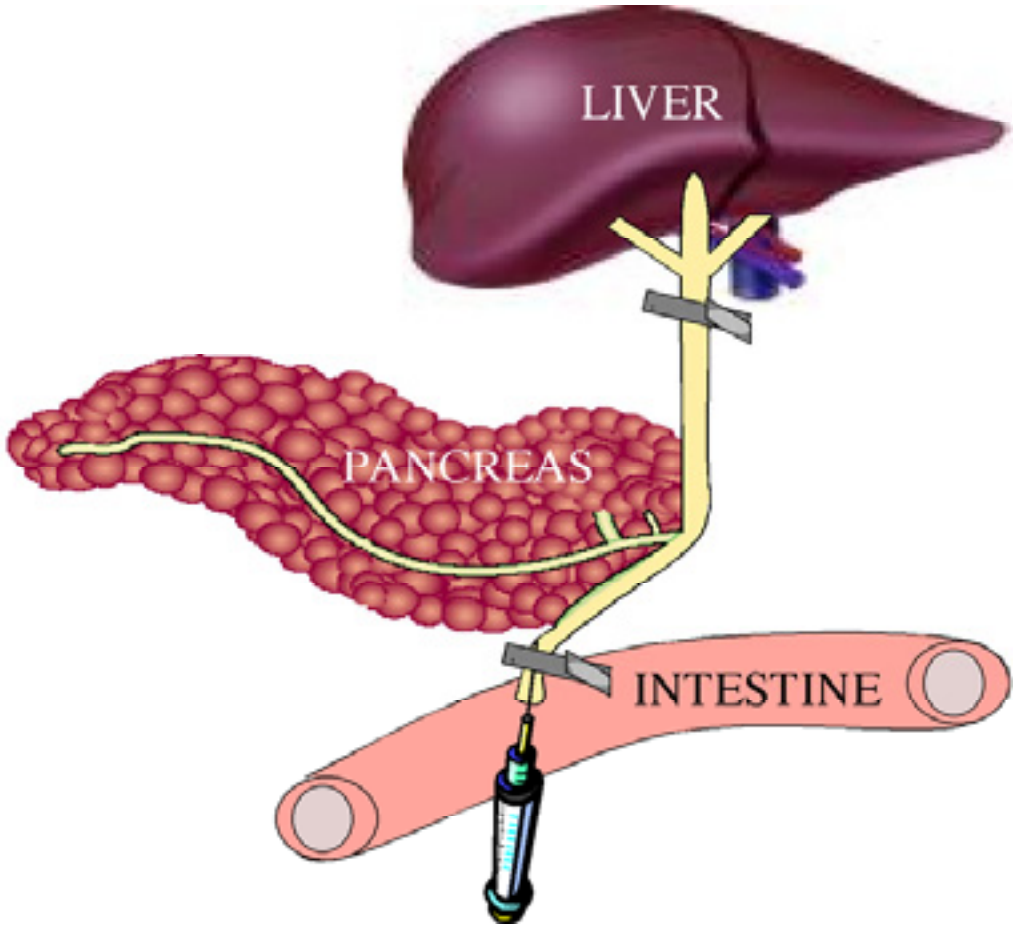


Figure 29. Schematic representation of intraductal delivery of AAV. The viral vector solution was injected using a syringe with a 30G needle. One vascular micro-clamp was situated in the common bile duct near the liver and a second clamp was used to fix the needle of the syringe and avoid the viruses to enter into the intestine. A detailed explanation of the procedure is described in Material and Methods.

We injected $1.5x10^{10}$ vg/mouse of AAV8 CAG-GFP and we observed high transduction of the exocrine and endocrine pancreas (Fig. 30). Small islets resulted in almost 100% transduction, whereas large islets were more transduced in the periphery than in the core (Figure 30). However, although the bile duct was clamped before branching at the liver edge, notable expression of the marker genes was found in the hepatic tissue (Fig. 31). These data suggested that selective promoters should be used to avoid expression of the therapeutic gene in undesired tissues. Nevertheless, intraductal delivery of the AAV8 vectors was the most efficient method to transduce the pancreas compared with intravenous or intraperitoneal delivery.

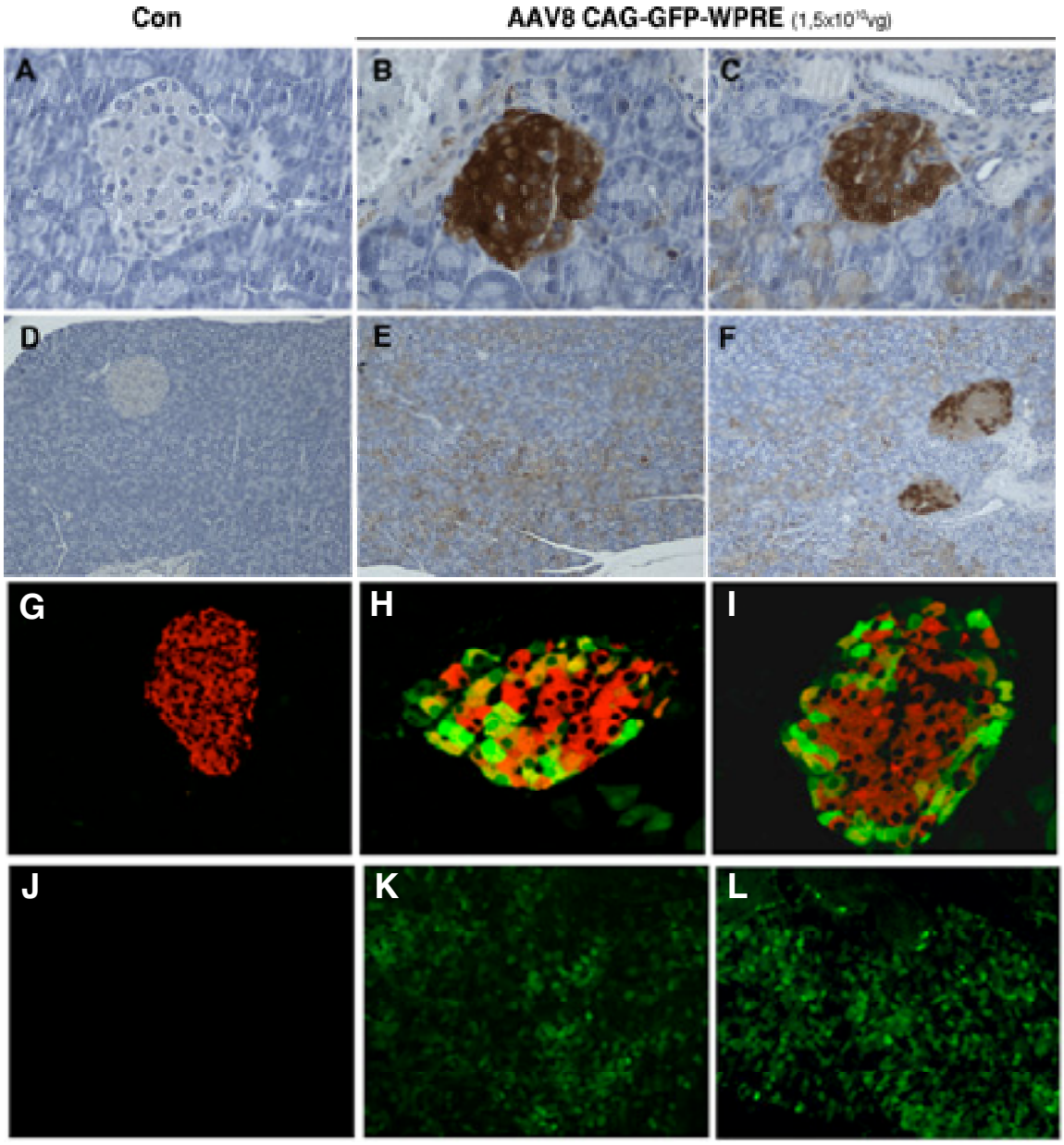


Figure 30. Pancreas transduction by intraductal delivery of AAV8 vectors. Immunohistochemical analysis showed that both exocrine (**E,F,K,L**) and endocrine (**B,C,G,I**) pancreas expressed GFP (brown in **A-F**, and green in **G-L**)) after ductal injection of AAV8 vectors. Immunohistochemical detection of GFP in PBS injected animal (con) was completely negative in the pancreas (A,D,G,J). Original magnification; x400 (**A-C,H,I**), x100 (**D-F, J-L**), x200 (**G**).

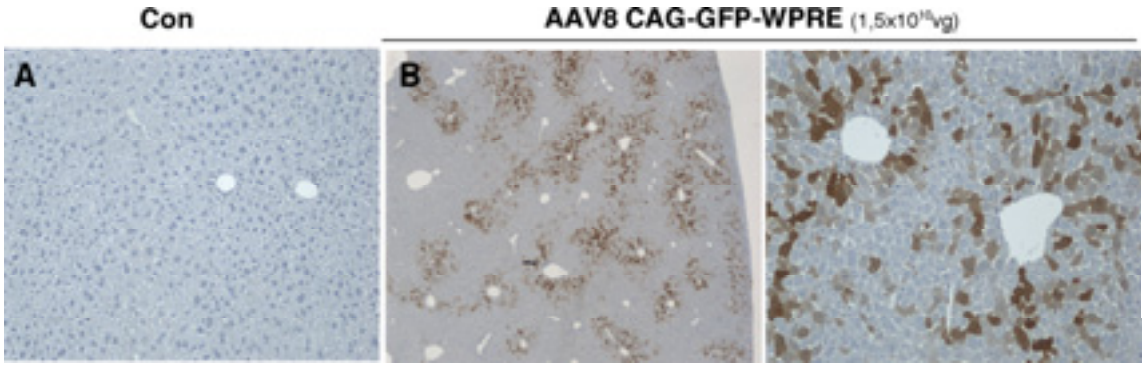


Figure 31. Liver transduction by intraductal delivery of AAV8 vectors. Immunohistochemical analysis showed that liver expressed GFP (brown) after ductal injection of AAV8 vectors. Immunohistochemical detection of GFP in PBS injected animal (con) was completely negative in the liver. Original magnification; x100 (A), x40 (B), x200 (C).

2. GENE TRANSFER OF IGF-I TO THE PANCREAS IN VIVO USING VIRAL VECTORS.

Expression of IGF-I in ß-cells of transgenic mice reverted type 1 diabetes by a mechanism involving ß-cell replication, neogenesis and counteraction of apoptosis (George *et al.*, 2002). In addition, ß-cell expression of IGF-I in transgenic mice expressing IFN-ß in insulin producing cells protects from lymphocytic infiltration and development of diabetes (Casellas et al. manuscript submitted). These encouraging results together with the successful pancreatic gene transfer described above using viral vectors led us to propose a new gene therapy approach for type 1 diabetes based on genetic manipulation of the pancreas by using viral vectors to deliver an IGF-I transgene.

2. 1. Generation of IGF-I expressing viral vectors.

The results obtained with marker genes indicated that systemic administration of adenoviral vector in mice with the hepatic circulation clamped and retrograde pancreatic duct delivery of AAV8 vectors were the most efficient methods for pancreatic transduction *in vivo*. Thus, we focused on using HD-Ad and AAV8 vectors to deliver the IGF-I gene.

2. 1.1. Generation and testing IGF-I-expressing helper-dependent vectors.

The first step in generating the IGF-I-expressing HD-Ad was the cloning of the murine IGF-I cDNA into the MCS of the PTG6600 plasmid to generate the expression cassette. In this cassette, the CMV promoter drives the expression of the IGF-I. Thereafter, the expression cassette was cloned by blunt-end ligation into the *NotI* restriction site of the pSTK129 plasmid which contained the ITRs, packaging signaling and stuffer DNA from HPRT and C346 as described in section 1.2.1. The cloning steps are summarized in Fig. 32. The generation of infectious particles starting from plasmid was carried out as described in Material and Methods (section 6). The resulting viral stock had a titer of $2x10^{11}$ pp/ml.

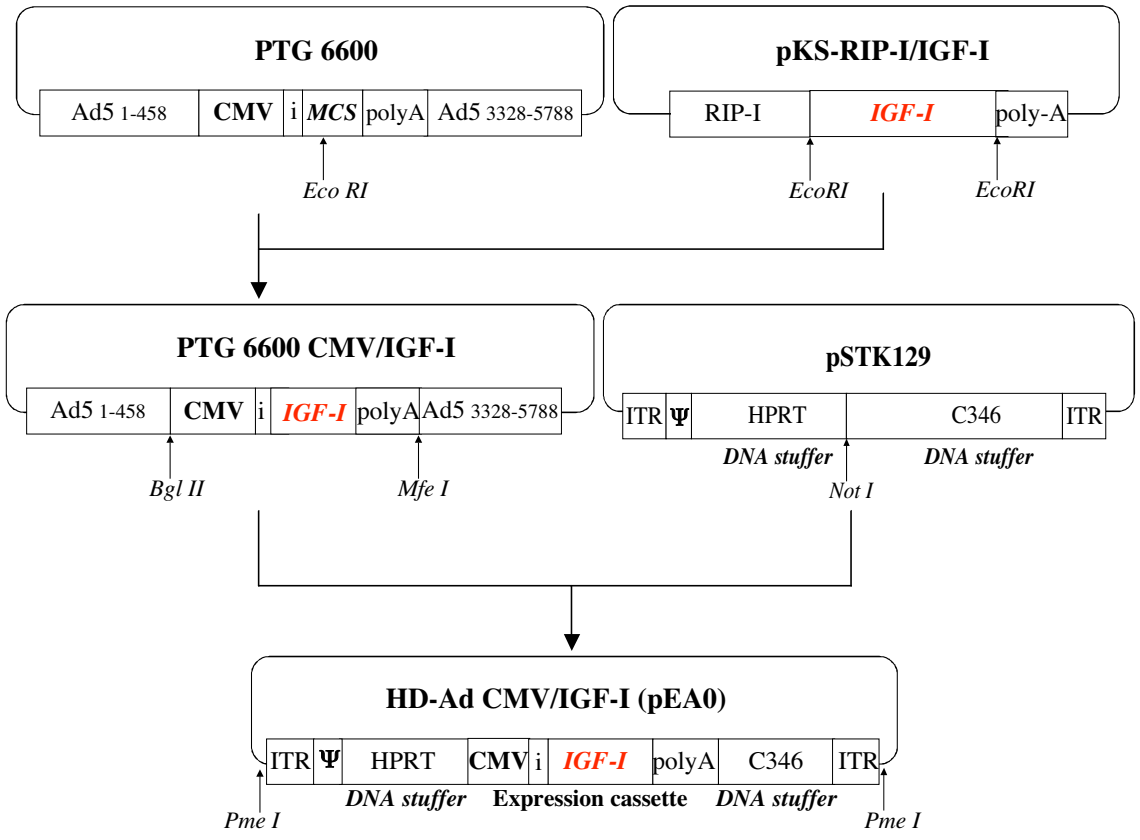


Figure 32. Cloning steps to generate the HD-Ad vector carrying the CMVIGF-I expression cassette. ITR=inverted terminal repeats, i=intron, polyA=polyadenilation signal, Ψ=packaging signal, MCS= Multi Cloning Site.

In order to test the functionality of this vector we infected $5x10^6$ A549 cells with 2 µl of the viral preparation. After 48h total RNA from the cells was isolated and Northern blot was performed using the IGF-I cDNA probe. The ribosomal protein S26 (Rps) was used to document equal loading. High expression of IGF-I mRNA was detected in cells transduced with HD-Ad CMV/IGF-I vectors (Fig. 33), validating the infectivity of the vector and the functionality of the expression cassette.

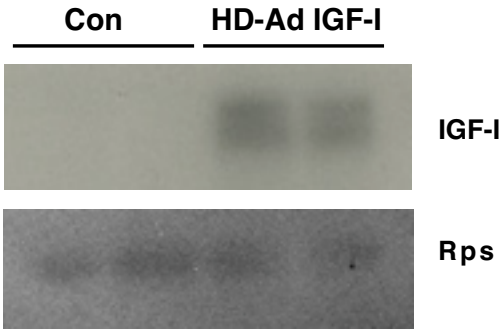


Figure 33. Expression of IGF-I in A549 cells transduced with HD-Ad CMV/IGF-I. Total cellular RNA was obtained from HD-Ad CMV/IGF-I transduced and non-transduced (con) cells and the Northern blot was performed as indicated in Material and Methods.

2. 1.2. Generation and testing IGF-I expressing AAV8 vectors.

To generate these vectors we took advantage of a plasmid generated previously in our laboratory containing the AAV ITRs, CAG promoter, murine IGF-I cDNA and WPRE. The scheme of the vector is shown in Fig. 34.

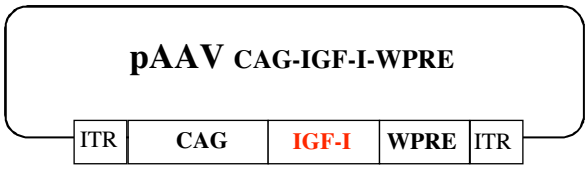


Figure 34. Schematic representation of AAV8 CAG-IGF-I-WPRE vector. ITR=inverted terminal repeats. WPRE=Woodchuck Posttranscriptional Regulatory Element.

This viral genome was used to generate the AAV8 particles. The procedure is described in Material and Methods (section 7) and the resulting viral preparation had a titer of $4x10^{11}$ vg/ml. To determine the *in vivo* functionality of these vectors, 30µl of the viral preparation were injected into the *tiabialis cranialis* muscle of a mouse. Fifteen days later the RNA was extracted from the muscle and Northern blot analysis was performed. We decided to directly test these vectors *in vivo* because high efficiency muscle transduction had been described using the AAV8 capsid (Wang et al. 2005; Nakai *et al.*, 2005). High expression of the IGF-I transcript was observed in the injected muscle compared with control (non-injected) muscles two weeks after vector administration (Fig. 35). This analysis demonstrated both the infectivity of the particles and the strength of the expression cassette.

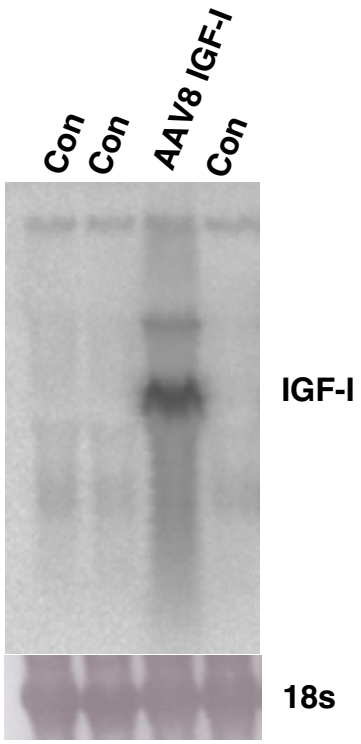


Figure 35. Expression of IGF-I in the muscle of mice injected with AAV8 CAG-IGF-I vector ($1.2x10^9$ vg/mouse). Total RNA from tiabialis cranialis muscle was obtained from injected and non-injected (con) cells and Northern blot was performed as indicated in Material and Methods.

2.2. *In vivo* administration of IGF-I expressing viral vectors into the pancreas.

To evaluate the functionality of the AAV vectors *in vivo* in the pancreas we injected $4x10^{10}$ vg/mouse of AAV8 CAG-IGF-I-WPRE via the pancreatic duct. Fifteen days after vector administration, mice were sacrificed and pancreata were fixed and embedded in paraffin. IGF-I was detected by immunohistochemical analysis in the islets of AAV-treated mice, however, no detection was observed in untreated mice (Fig. 36A). Since IGF-I is secretable protein, it is possible that very low levels of expression in normal islets accounted for the lack of immunostaining. These results suggested that IGF-I was detectable only when it was highly expressed and demonstrated that these AAV vectors were fully competent in terms of cell transduction and gene expression (Fig. 36).

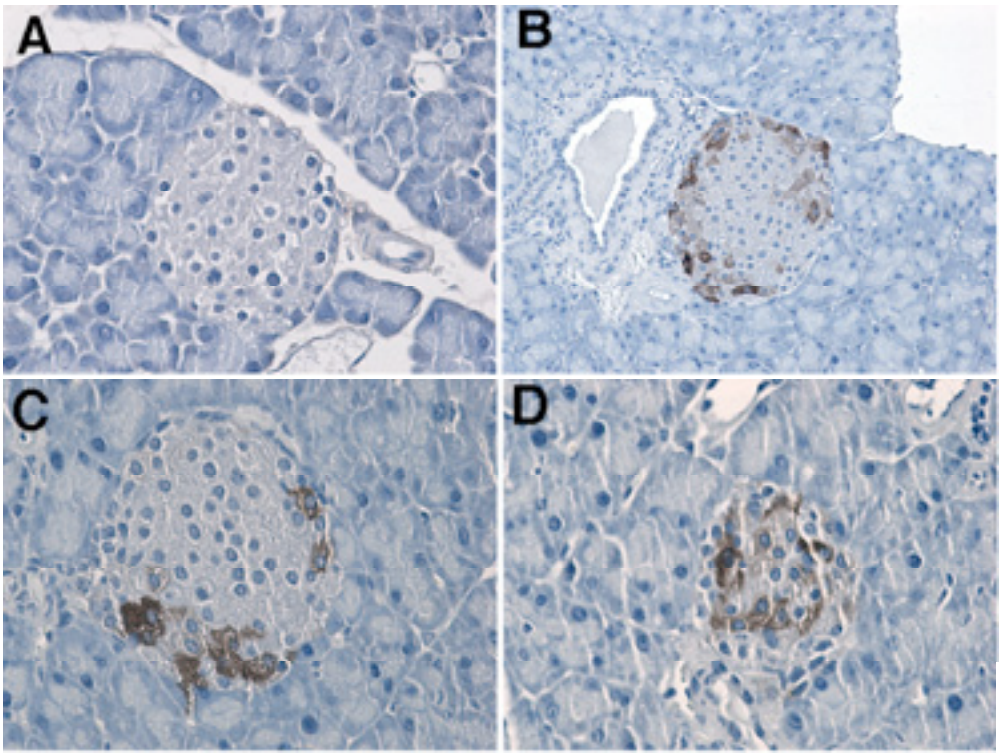


Figure 36. Immunohistochemical detection of IGF-I in the pancreas of mice injected intraductally with AAV8 CAG-IGF-I-WPRE ($4x10^{10}$ vg/mouse). No immunostaining was observed in non-injected mice (**A**) while IGF-I protein was detected in AAV8-injected mice (**B-D**). Original magnification x400 (**A,C,D**) x200 (**B**).

3. IN VIVO GENE TRANSFER TO HEALTHY AND DIABETIC CANINE PANCREAS

The number of gene therapy clinical trials increases every year, and promising results have been obtained treating several diseases. However, type 1 diabetes is far from being successfully targeted with gene therapy since genetic manipulation of the pancreas *in vivo* in large animal models remains elusive. Taking into account the anatomy and vascularization of the canine pancreas, we hypothesized that a clamp of the pancreatic circulation could be applied *in situ* and vectors could be injected directly into the pancreatic vessels. Therefore, we examined the ability of adenoviral vectors to transfer genes into the pancreas of dogs by using this approach.

3.1. Experimental design used for adenovirus administration to canine pancreas

Since blood supply to the pancreas results from multiple vessels, clamps at several sites were established to achieve blood stasis. The following vessels were clamped before vector administration: celiac, splenic, gastroduodenal, gastroepiploic and cranial and caudal pancreaticoduodenal arteries. As the veins parallel the arteries, clamps occluded both types of vessel. Once the clamp was established, a solution containing adenoviral vectors was infused into the pancreaticoduodenal vein. Figure 37 shows the canine pancreas and the pancreaticoduodenal cannulation and Figure 38 shows a schematic representation of the clamping sites.

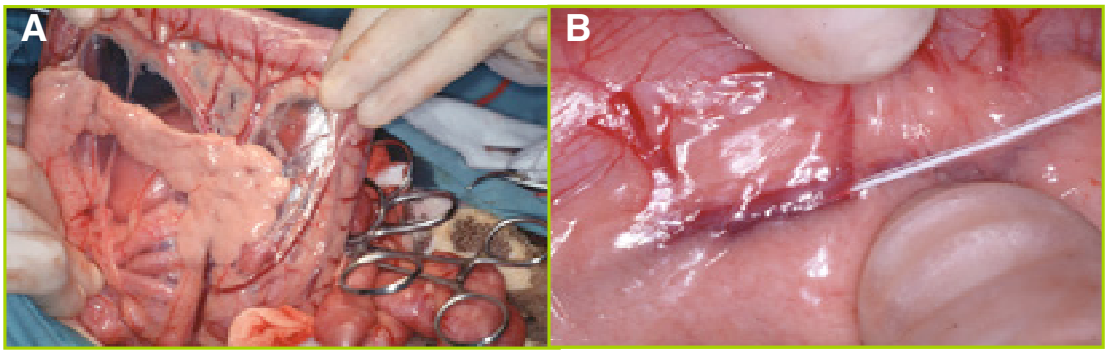


Figure 37. Pictures of the canine pancreas and the cannulation of the pancreaticoduodenal vein.

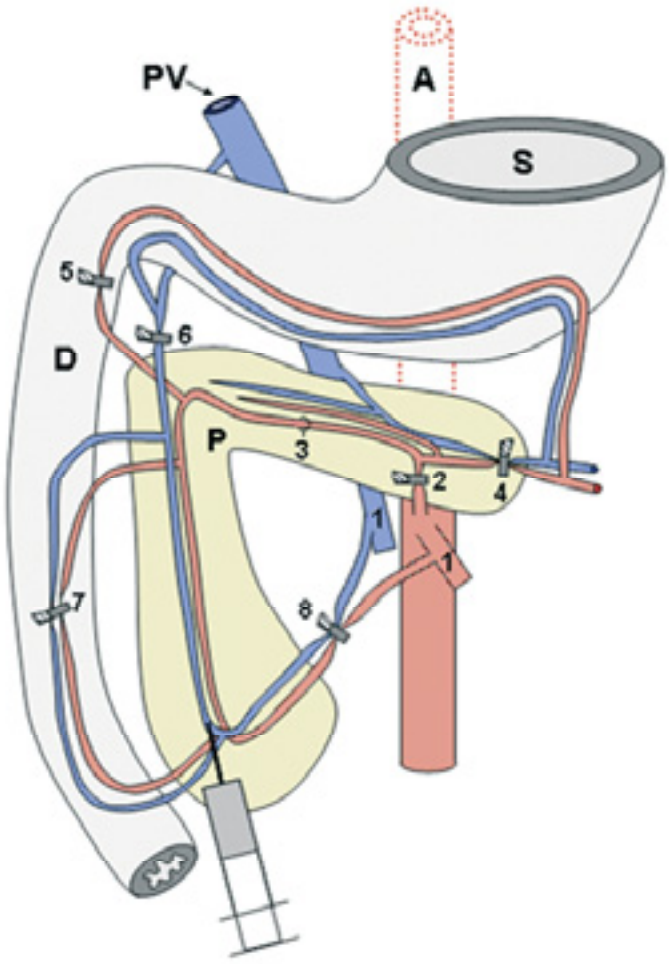


Figure 38. Scheme of canine pancreatic circulation showing the clamping sites. Stomach (S), Duodenum (D), Pancreas (P), Aorta (A), Portal vein (PV). The arterial system is colored in red and venous system in blue. 1: Cranial mesenteric artery and vein. 2: Celiac artery. 3: Hepatic artery. 4: Splenic artery and vein. 5: Right gastroepiploic artery. 6: Gastroduodenal vein. 7: Cranial pancreaticoduodenal artery and vein. 8: Caudal pancreaticoduodenal artery and vein. The syringe shows the site of virus injection.

3.2. Tranduction of the pancreas.

A total dose of $2x10^{10}$ IU/dog of adenoviral vector carrying the ß-galactosidase marker gene under the control of the CMV promoter was injected in the pancreaticoduodenal vein and the clamp was applied for 10 min. The dogs were euthanasied five days later. To study the distribution of the ß-gal expressing cells the pancreas was sectioned into four pieces, named P1, P2, P3 and P4 (Fig. 39A). Five days after vector injection into the pancreatic-duodenal vein, ß-gal was expressed throughout the pancreas (Fig 39C). However, ß-gal expression was higher in P1 and P2, which were closer to the site of vector injection. Transduction was lower in P3 and much lower in P4, where only a few cells were ß-gal positive (Fig 39C). However, while efficient transduction was achieved when viruses were injected through the pancreatic-duodenal vein in clamped dog pancreas, in non-clamped pancreas transduction was not observed (Fig. 39B). We have quantified the percentage of positive cells in histological sections stained for ß-gal. In the whole pancreas 1.7 ± 0.7% of cells were transduced (P1: 3.3±1,7%; P2: 1.7±1; P3: 0.3±0,2%; and P4: 0.01±0,008% n=3).

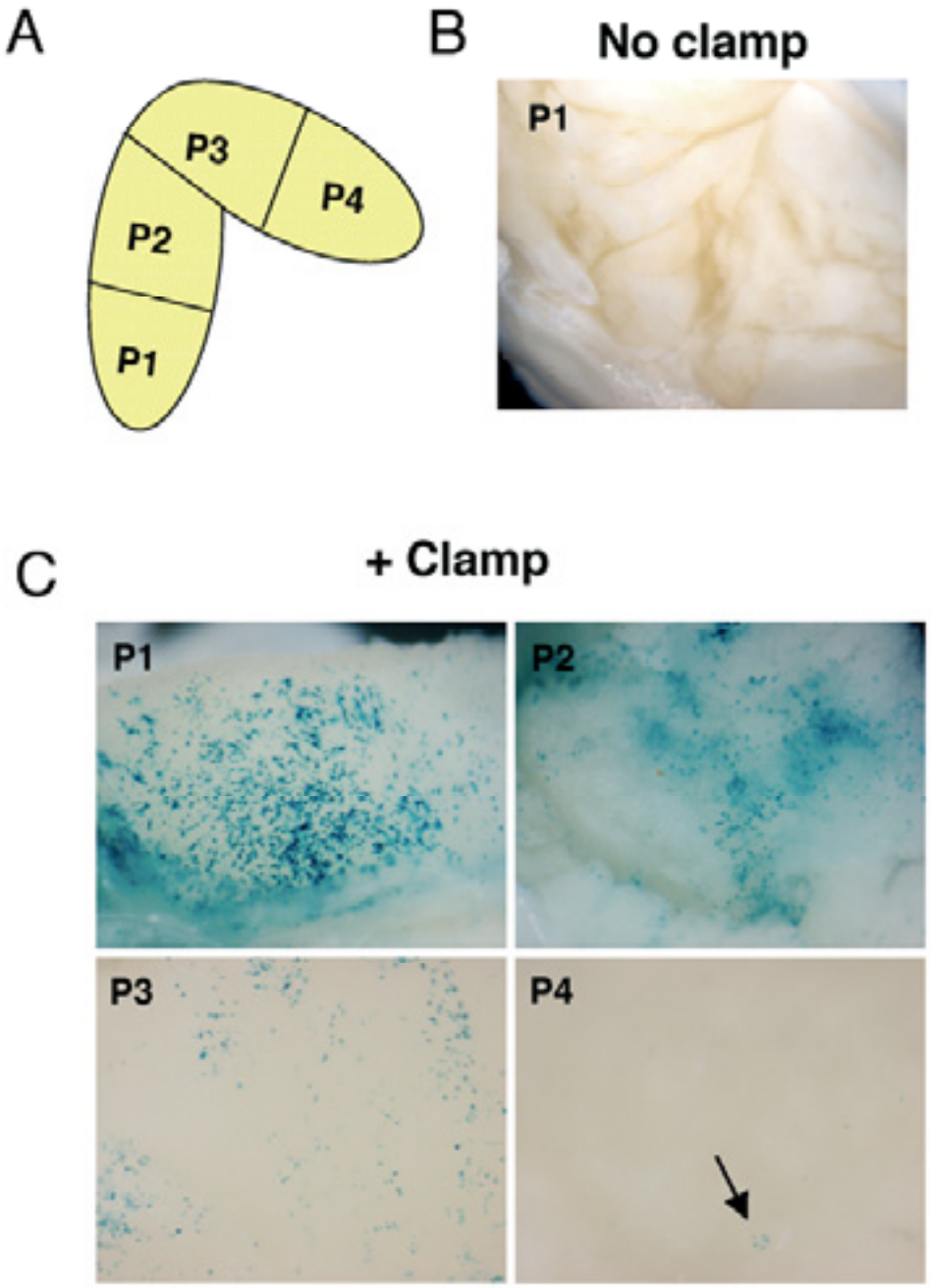


Figure 39. Pancreas gene transfer of dogs that underwent pancreatic circulation clamp. (**A**) Pancreata from clamped dogs were removed 5 days after adenoviral vector administration and divided into 4 parts: P1, P2, P3 and P4 as indicated. (**B**) No X-gal staining was detected in pancreas of non-clamped dogs after *in toto* analysis. (**C**) X-Gal staining of the pancreas revealed that adenoviral transduction was mainly extended from P1 to P3, although few transduced cells in P4 (arrow) were also observed. Original magnification x25.

ß-gal expression after injection to the pancreaticoduodenal artery was similar to the expression observed following injection into the pancreaticoduodenal vein (Fig. 40). These results suggest that both routes of administration, either vein or artery, may be used when blood stasis is established within the pancreas.

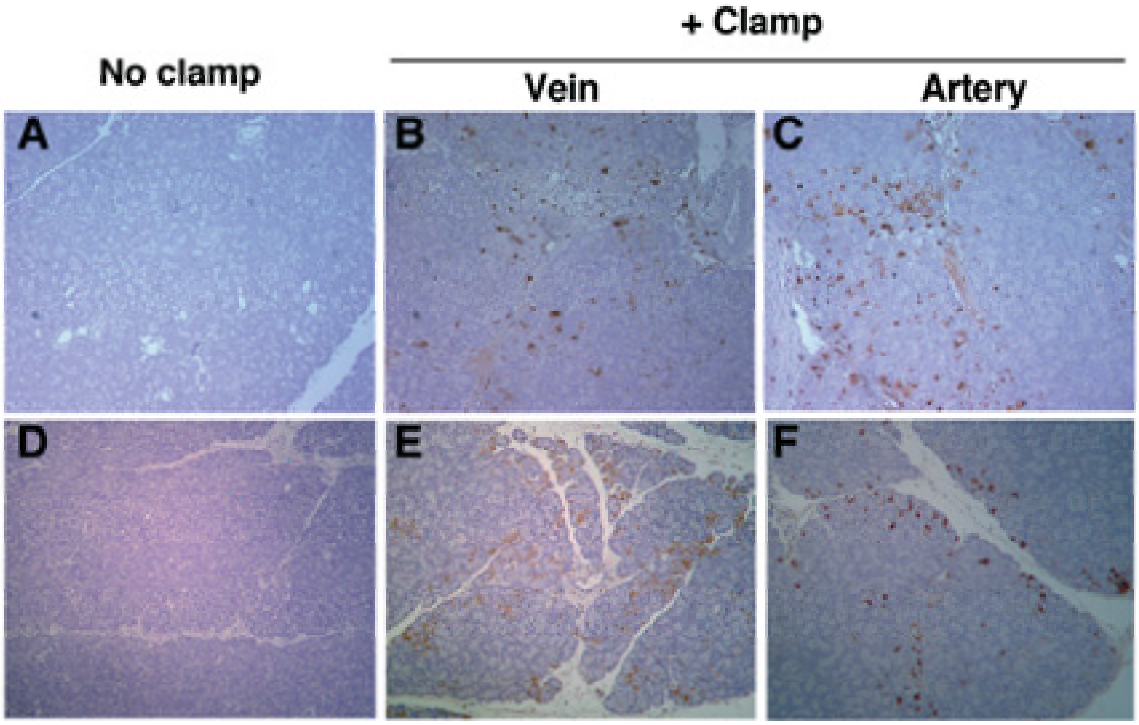


Figure 40. Immunohistochemical analysis of ß-gal expression in the pancreas. Non-clamped (**A,D**) and clamped (**B,C,E,F**) dog pancreata were analyzed 5 days after vector administration. (**A,D**) No ß-gal expression was observed in non-clamped animals. (**B,C,E,F**) Pancreas gene transfer was achieved in clamped dogs both when the injection of the adenovirus was performed by the vein (**B,E**) or by the artery (**C,F**). Original magnification x100.

3.3. Transduced cell types.

ß-gal-positive cells were located near the lobe septum (Fig. 41A), especially in the connective tissue forming the septa (Fig. 41B,C). Furthermore, acinar cells showed prominent ß-gal expression (Fig. 42). In addition, positive nuclei for ß-gal staining were detected in ducts of clamped animals (Fig. 42). After both venous and arterial delivery

of the vectors we observed that acinar cells were preferentially transduced. Neither acinar nor ductal cells expressed ß-gal in dogs that were not clamped (Fig. 42A,C).

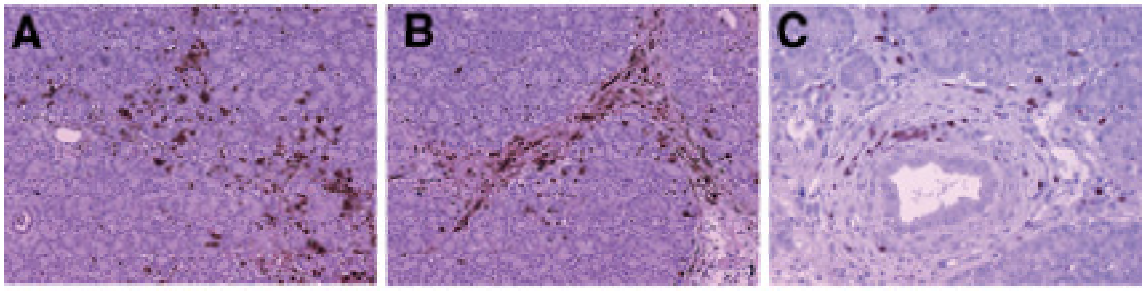


Figure 41. (A-C) Connective tissue in the septa was efficiently transduced. Septa divide the pancreas into lobules, bounded by connective tissue. Between the lobules, connective tissue surround the larger ducts, blood vessels, and nerve fibers. (**A**) ß-gal positive cells were located around septa can be observed (x100). (**B**) Adenovirus transduction of the connective tissue in a longitudinal section (x100). (**C**) ß-gal positive cells in the connective tissue surrounding pancreatic duct in a cross-section (x200).

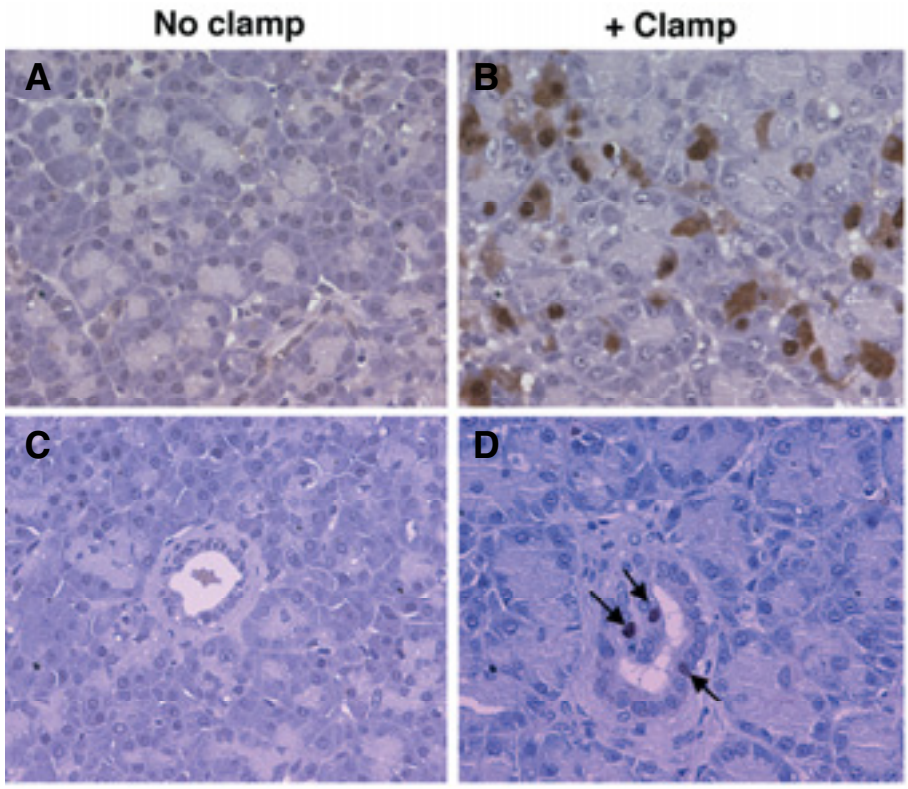


Figure 42. Gene transfer to acinar and ductal cells of dog pancreas. ß-gal immunostaining was performed in pancreatic sections 5 days after adenovirus injection. (**B**) Acinar cells were highly transduced by the adenovirus in clamped dog. (**A**) In contrast, exocrine pancreas was not transduced in dogs without clamp. (**D**) Ductal cells were also transduced (arrows) by the adenovirus in animals that underwent pancreatic clamp. Original magnification x400.

The distribution and shape of dog islets was also analyzed, by insulin and glucagon immunostaining of pancreatic sections (Fig. 43A-F). Canine pancreas contained large number of small islets homogeneously distributed throughout the section (Fig. 43D). The mouse pancreas, in contrast, contained significantly fewer islets (dogs, 11.7± 1.1 islets/mm^2 of pancreas *vs.* mice, 1.47± 0.1 islets/mm^2 of pancreas, $p<0.05$), although they were larger (mouse islet area, 3878 ± 190 μm^2 *vs.* dog, 1075 ± 96 μm^2, $p<0.05$) (Fig. 43A). In mouse islets, glucagon-producing α-cells were mainly distributed at the periphery, whereas insulin-producing ß-cells were seen in the core (Fig. 43B,C). In contrast, dog islets showed irregular α- and ß-cell distribution (Fig. 43E,F). Furthermore, scattered endocrine cells, either α or ß cells, were observed throughout the dog pancreas (Fig. 43D-F).

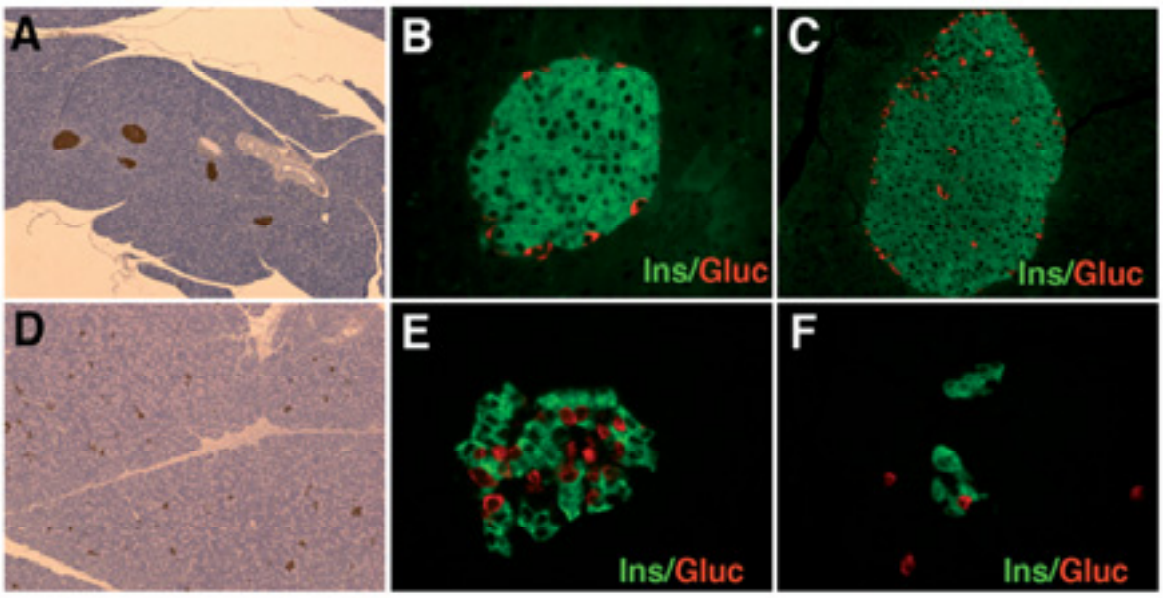


Figure 43. Analysis of dog endocrine pancreas. The distribution of insulin-producing cells in canine islets was determined in paraffin sections and compared with that of mouse islets (**A-F**). Mouse pancreas (**A**) showed small number of larger islets. Islet architecture was examined by double immunostaining of insulin (green) and glucagon (red). In mouse islets, ß-cells reside in the core whereas the α-cells are located in the periphery (**B,C**). Altered distribution of ß and α cells was observed in canine islets (**E,F**). Furthermore, small groups of α and ß cells distributed throughout the pancreas can also be observed (**F**). Original magnification x40 (**A,D**), x200 (**C**) and x400 (**B,E,F**).

Double immunostaining revealed ß-cells in the islets that were positive for insulin and ß-gal (Fig. 44). Furthermore, many ß-gal positive cells were also detected around the islets, suggesting that adenovirus transduction of dog pancreas was not selective for a specific cell type.

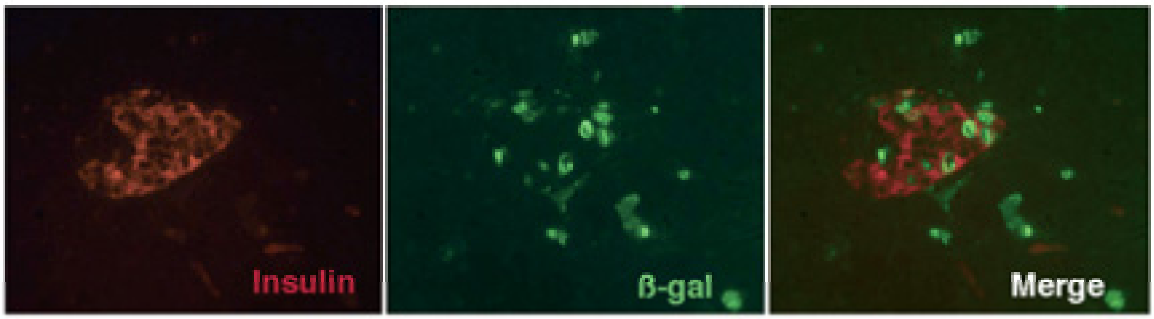


Figure 44. Endocrine pancreas transduction. Insulin and ß-gal double immunostaining was carried out in transduced pancreas. ß-cells expressing ß-gal can be observed (x400).

When the clamp was released adenovirus escaped from the pancreatic circulation, into the general circulation and was taken up by the liver. However, ß-gal expression in the liver was similarly low in clamped and non-clamped animals (Fig. 45).

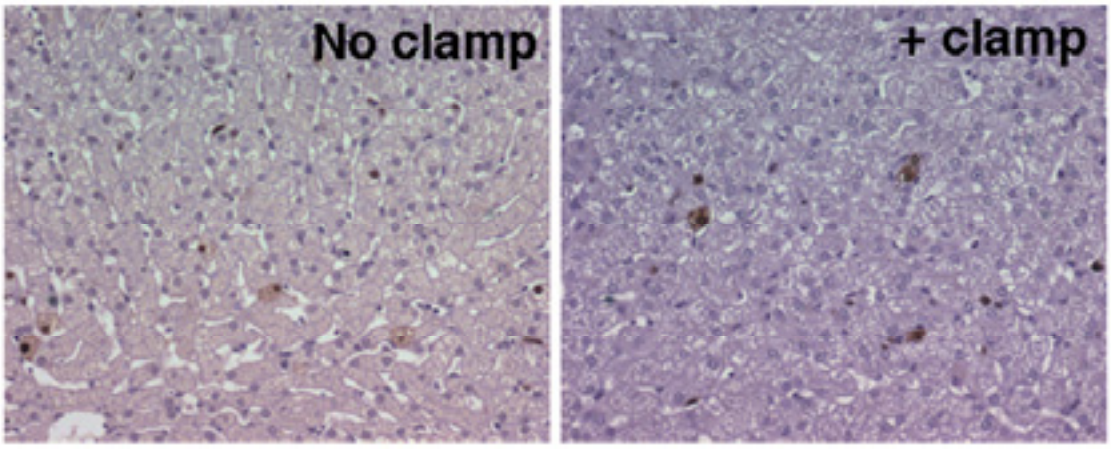


Figure 45. Liver transduction. Transduction level was similar in clamped and non-clamped animals. Equal amounts of viral vectors were used. ß-gal immunostaining of liver sections. Original magnification x200.

3.4. Evaluation of pancreatic and liver damage.

To evaluate pancreas and liver damage caused by the clamp and/or the adenoviral vectors, serum samples were taken the day before surgery (control value) and subsequently one, two, five and fifteen days thereafter. Amylase and lipase are common markers of pancreatic damage, and alanine-amino transferase (ALT) of liver damage. ALT, amylase and lipase levels were within the normal range five days after surgery (Table 2). Only one of the 11 dogs that were clamped showed amylase and lipase levels above normal values five days after the surgery. However, no symptoms of severe pancreatitis, such as vomiting or diarrhea, were observed.

	ALT (U/l)				Amylase (U/l)				Lipase (U/l)			
	d0	**d1**	**d5**	**d15**	**d0**	**d1**	**d5**	**d15**	**d0**	**d1**	**d5**	**d15**
Dog 1	26	29	96	-	1162	1248	1173	-	111	134	72	-
Dog 2	41	36	75	-	1129	825	1166	-	87	21	76	-
Dog 3	96	65	61	-	1173	1471	1948	-	72	72	83	-
Dog 4	63	-	31	-	832	-	1170	-	196	-	126	-
Dog 5	39	41	101	-	690	839	501	-	87	277	265	-
Dog 6	87	92	78	-	1063	849	850	-	257	93	76	-
Dog 7	25	-	29	-	1096	-	1299	-	225	-	216	-
Dog 8	44	-	1395[a]	-	1041	-	4000[a]	-	111	-	6628[a]	-
Dog 9	34	-	25	27	938	-	1057	911	81	-	53	52
Dog 10	34	-	36	29	704	-	554	600	57	-	48	41
Dog 11 (unclamped)	24	-	23	-	637	-	713	-	107	-	62	-
Dog 12 (diabetic)	-	-	-	-	1385	1299[b]	-	-	263	211[b]	-	-

Table 2. Serum enzyme activity after surgery. Serum samples were taken the day before surgery (control value) and one, two, five and fifteen days thereafter. Normal values: ALT: 21-102 U/l; amylase: 185-2000 U/l; lipase: 13-200 U/l. Dogs 1 to 10 were healthy dogs that underwent pancreatic clamping. Dog 11 was unclamped. Dogs 1-8 were injected with $2x10^{10}$ IU of adenoviral vectors. Dogs 9 and 10 were injected with saline. Dog 12 was a diabetic dog that underwent pancreatic clamping. [a] Parameter out of the upper range. [b] Serum value of day 2 instead of day 1 after the surgery. (-) Not determined.

3.5. Gene transfer to diabetic canine pancreas.

To test the feasibility of this gene transfer approach in diabetic animals, one dog was treated with a single intravenous injection of streptozotocin (STZ) and alloxan mixture to induce experimental diabetes (Anderson *et al.*, 1993). Since destruction of ß-cells led to massive insulin release few hours after the STZ/alloxan injection, the dog developed hypoglycemia. To maintain normoglycemia during this period, the dog was monitored and glycemia controlled by glucose infusion. Two days after STZ/alloxan injection, hyperglycemia (>250mg/dl) was detected, and blood glucose levels were maintained below 300 mg/dl by subcutaneous injections of 8 units of soluble insulin (Fig. 46). Afterwards, surgery was carried out in the diabetic dog, the pancreatic circulation was clamped and $2x10^{10}$ IU of AdCMV/ßgal were injected into the pancreaticoduodenal vein. Insulin immunostaining confirmed the loss of ß-cells in the diabetic pancreas (Fig. 47A,B). This was consistent with the decrease in serum insulin levels (data not shown). ß-gal was expressed throughout the pancreas in the diabetic dog (Fig. 47D), mainly in acinar cells and similarly to healthy dogs (Fig. 47C). Thus, these results indicate that surgery and vector administration were feasible under diabetic conditions, and that the pancreas was efficiently transduced.

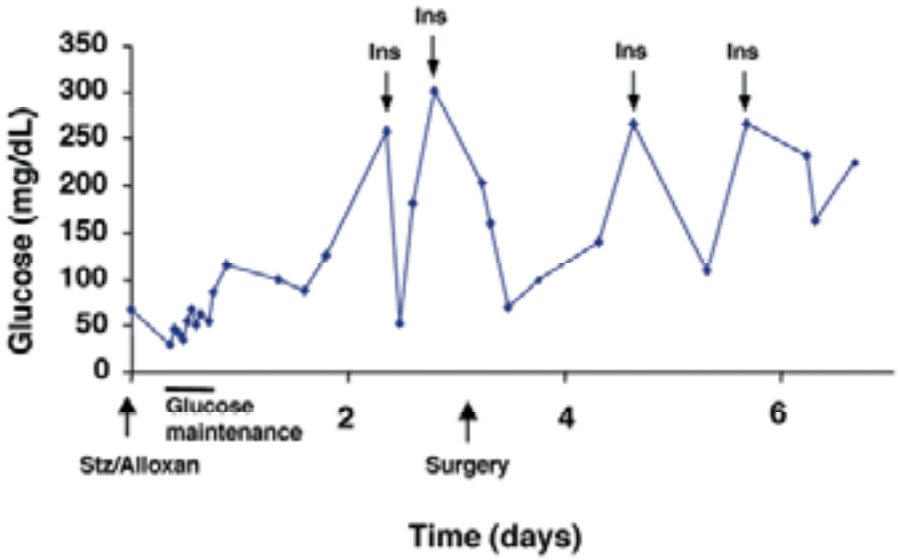


Figure 46. Diabetes induction. Experimental diabetes was induced by streptozotocin /alloxan injection. A few hours afterwards, the animals developed hypoglycemia due to massive destruction of ß cells and insulin release. Glycemia was controlled by glucose infusion. When hyperglycemia was observed, the dog was treated with subcutaneous injection of 8 units of soluble insulin (Ins). Surgery and vector administration was performed three days after diabetes induction.

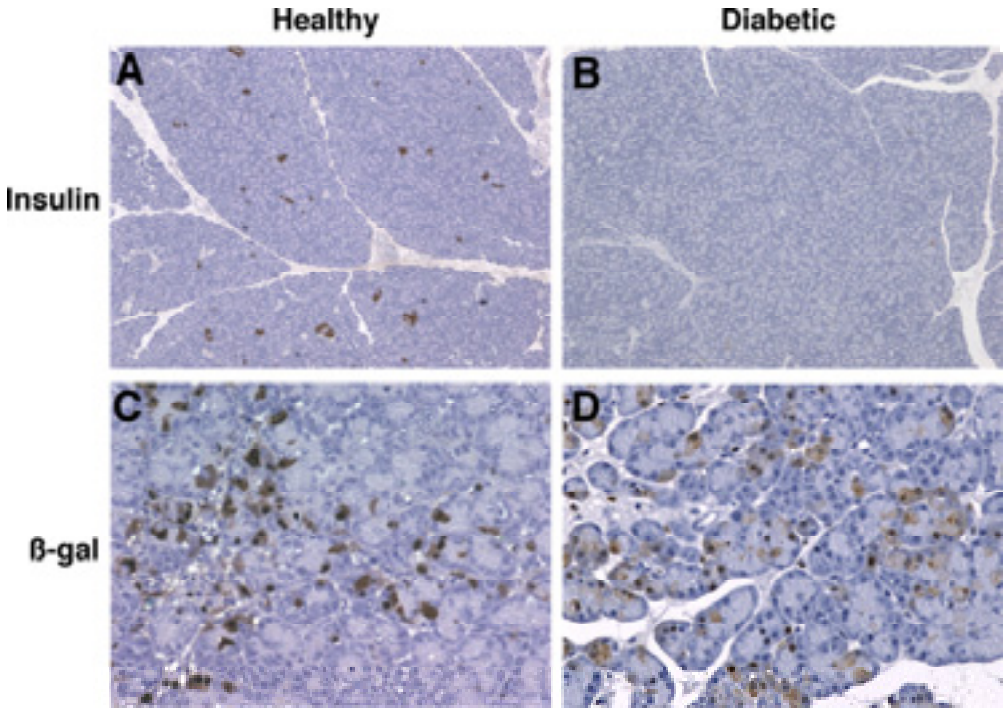


Fig 47. Gene transfer to diabetic canine pancreas. (**A,B**) Reduction of ß-cell mass was observed in the diabetic dog after insulin immunostaining of pancreatic sections. (**C,D**) Gene transfer was also achieved in diabetic pancreas. Pancreatic sections of healthy (**C**) and diabetic (**D**) dogs immunostained with ß-gal antibody (brown) are shown. Original magnification 40X (**A,B**), and 200X (**C,D**).

DISCUSSION

In this study we demonstrated that gene transfer to mouse ß-cells *in vivo* can be achieved by using systemic delivery of adenoviral vector bypassing the hepatic circulation. In contrast to direct pancreatic injection with adenoviral vectors that leads to transduction of few endocrine cells (Raper and DeMatteo, 1996; McClane *et al.*, 1997a; McClane *et al.*, 1997b; McClane *et al.*, 1997c; Wang *et al.*, 2004a), we found that about 70% of pancreatic islets showed ß-gal expression with an average of about 20% of the cells within positive islets being transduced. The fact that ß-cells were preferentially transduced compared with acinar cells might result from both the high vascularization of islets and the fenestrated structure of islet capillaries (Lammert *et al.*, 2003). Similarly, renal glomeruli, which also contain fenestrated capillaries, are specifically transduced by systemic delivery of adenovirus in portal clamped animals (Ye *et al.*, 2002).

Despite the high transduction observed in islets seven days after adenovirus administration, a significant decrease in ß-gal expression was observed at 14 and 30 days post-transduction. It has been described that first-generation adenoviral vectors could induce immune response against transduced cells, since viral proteins are expressed, although at low levels (Liu and Muruve, 2003). When the incidence and severity of the lymphocytic infiltration of islets was measured, we found that only a small percentage of ß-gal-expressing islets (up to 20%) presented perinsulitis, which did not progress to severe insulitis one month after adenoviral vector injection.

Large animals are more representative models of human diseases, especially the dog, but canine pancreas and murine pancreas are different (see Results 3.3). To validate the results obtained in mice in a large animal model, and taking into account the anatomy and vascularization of the dog pancreas, we hypothesized that a clamp of the pancreatic circulation could be applied *in situ* and vectors could be injected directly into the pancreatic vessels. We speculated that this technique might increase pancreas transduction and avoid liver damage. In fact, injection of adenoviral vectors into canine pancreatic circulation led to efficient *in vivo* gene transfer throughout the pancreas. Most of the ß-gal immunostaining was seen in acinar cells, in connective tissue surrounding the main blood vessels and in the islets and ducts. However, after intravascular injection, the pattern of transduction differed from the pattern seen in mice, in which ß-cells were preferentially transduced rather than acinar cells, because of the high vascularization of islets and the fenestrated structure of islet capillaries (Lammert *et al.*, 2003). The presence of endocrine cells scattered throughout the canine pancreas, and morphological differences in the islets might explain the discrepancies between dogs and mice. Furthermore, the local clamp induced blood stasis in the dog pancreas, limiting the distribution of the adenoviruses. In addition, dogs and rodents also differ in their lobe structure and blood supply. In dogs, connective tissue surrounds the blood vessels, ducts and nerve fibers, as in human pancreas. Since vectors were injected intravascularly, ß-gal expression was higher in the connective tissue, acinar cells and pancreatic islets near the septa than in those located in the center of the lobe. These data suggested that vectors escaped from the blood vessels and did not diffuse, probably because of the blood stasis.

Long-term expression could not be expected from these experiments since first-generation adenoviruses were used. Thus, dogs were euthanasied five days after adenoviral vector administration, when maximal expression of the delivered gene was expected. To avoid undesirable effects of first-generation adenoviral vectors that might lead to transient expression of the genes of interest, helper-dependent adenoviral vectors (HDAd) may be used (Kay *et al.*, 2001; Volpers and Kochanek, 2004; Alba *et al.*, 2005). These vectors minimize the host adaptive response and improve the efficacy and duration of gene transfer *in vivo* (Morral *et al.*, 1998; Morsy *et al.*, 1998; Schiedner *et al.*, 1998; Morral *et al.*, 1999; Balague *et al.*, 2000; Cregan *et al.*, 2000; Kim *et al.*, 2001; Maione *et al.*, 2001; Oka *et al.*, 2001; Zou *et al.*, 2001; Ehrhardt and Kay, 2002; Reddy *et al.*, 2002) (Gilbert *et al.*, 2002; Gilbert *et al.*, 2003; Dudley *et al.*, 2004; Fleury *et al.*, 2004; Pastore *et al.*, 2004; Wen *et al.*, 2004; Brunetti-Pierri *et al.*, 2005; Mian *et al.*, 2005; Toietta *et al.*, 2005). Therefore, in this study we also aimed to evaluate the ability of HD-Ad to transduce the pancreas in mice by bypassing the hepatic circulation in order to achieve long-term gene expression. Expression of the marker genes (either GFP or ß-gal) was found in pancreatic islets 7 days after the injection of the vectors. These results indicated that HD-Ad could be used for *in vivo* gene transfer to the pancreas. However, further experiments are needed to evaluate the duration of the HD-Ad-mediated transgene expression in this organ.

In addition to viral proteins, the expression of foreign genes, such as ß-gal and GFP, might have increased the immune response against transduced cells when using FG-Ad. In previous works (Chen *et al.*, 1997), expression of ß-gal in the skeletal muscle

of immunocompetent mice using HD-Ad resulted in a decrease in vector copy number as early as 28 days after injection, and complete loss of ß-gal expression in two out of three mice at 42 days. HD-Ad treated mice showed extensive inflammatory cellular infiltration consisting primarily of $CD4^+$ and $CD8^+$ lymphocytes, associated with the expression of the ß-gal (Chen *et al.*, 1997). Similarly, loss of expression was also observed using HDAd ß-gal vectors in lungs (Toietta *et al.*, 2003). Furthermore, since ß-gal expression in our experiments is controlled by the CMV promoter, an immune-mediated repression of the CMV promoter cannot be ruled out. It has been shown that interferon-γ, which is normally produced in the course of T cell-mediated immune response, can inhibit the CMV promoter activity (Harms and Splitter, 1995).

In addition to adenovirus, lentiviral and adeno-associated (AAV) viral vectors have been shown to infect islets *in vitro* (Giannoukakis *et al.*, 1999a; Flotte *et al.*, 2001) and have the potential to be used to transduce endocrine pancreas *in vivo* for long-term expression. Recent *in vitro* studies showed that Lymphocytic Choriomeningitis virus-pseudotyped lentiviral vectors can transduce insulin-secreting ß-cells with greater efficiency than Vesicular Stomatitis virus, Murine Leukemia virus, Ebola, Rabies or Mokola pseudotyped lentiviral vectors (Kobinger *et al.*, 2004). AAV-mediated gene transfer to murine islets *in vitro* using non-serotype-2 AAV capsids, such as 6, 8, 5 and 1, can also mediate efficient transduction of islet cells, with AAV6 and AAV8 being the most efficient serotypes (Wang *et al.* 2006). In addition, it was shown that AAV8 was more efficient than AAV2 in transducing the pancreas *in vivo* after direct injection, whereas AAV5 did not result in any detectable transgene expression (Wang *et al.*, 2004a). This study also showed that adenoviral vectors were more efficient than AAV8

in transducing the pancreas *in vivo*. However, the persistence of gene expression when using AAV vectors was longer when compared to adenoviral vectors, which elicited significant leukocyte infiltration and the subsequent loss of more than 90% of expression after 4 weeks (Wang *et al.*, 2004a). In addition, AAV8 vectors injected into mouse pancreatic duct led to efficient transduction of acinar cells, but with less than 5% of ductal cells and a minimal amount of ß-cells being transduced (Loiler *et al.*, 2005). However, the tropism of the AAV8 vectors to target the pancreas after systemic delivery remained unexplored. Therefore, in this study, we have examined the potential of AAV8-mediated gene transfer of the exocrine and endocrine pancreas *in vivo* by different routes of administration. Intravascular deliver of AAV8 vectors ($5x10^{10}$ vg/mouse) into general circulation of adult mice led to liver, but not pancreas transduction. The closure of hepatic circulation did not modify the tropism of the vector for the pancreas and no ß-gal expression was observed in pancreatic cells using AAV8 CMV/ß-gal vectors ($5x10^{10}$ vg/mouse) and portal clamp (30 min). In addition, we also examined tissue transduction after intraperitoneal injection of AAV8 vectors in neonatal mice (1 week, 2 week and 3 week-old). We observed high tropism of the vector for the heart and the liver, but no pancreatic transduction was observed in 1 or 2 week-old mice and $1.5x10^{10}$ vg/mouse. Few scattered acinar cells were found positive for X-gal staining when $5x10^{10}$ vg/mouse of AAV8 CMV/ßgal was injected in 3 week-old mice (data not shown). Nevertheless, the use of $7x10^{10}$ vg and the RIP-II/GFP specific construct did not result in any transduction of ß-cells. In contrast, the intraductal injection of the AAV8 vectors resulted in high transduction of the exocrine pancreas and remarkable transduction of the endocrine pancreas. Transduction of acinar cells might be used to

express and secrete proteins that may act in the surrounding islets in a paracrine manner.

During the writing of this thesis one study published by Wang et al. described experiments very similar to the ones we performed in this work. Several AAV serotypes were compared in terms of efficiency for *in vivo* gene transfer to the pancreas after delivery by different routes. In that study intravascular and intraperitoneal administration of AAV8 vectors led to efficient pancreatic gene transfer. However, very high doses of double-stranded (ds) AAV vectors (up to $2x10^{12}$ vg/mouse) were used (Wang *et al.*, 2006). It has been shown that double-stranded ds-AAV are much more efficient than single stranded (ss) vectors because the intracellular conversion of ss genome into ds is a limiting step for transgene expression (McCarty *et al.*, 2003; Wang *et al.*, 2003). Therefore, the combination of ds genome and high doses of the vectors resulted in pancreatic transduction. However, ds-AAV limits the number of therapeutic genes that can be cloned into this system, since these vectors accommodate expression cassettes only up to 2.5 kb instead of the ~4.7 kb accommodated by ss-AAV. In addition, the high doses of viral vectors used in that study ($2x10^{12}$ vg/mouse) are difficult to translate into clinical application since this dosage is equivalent to ~$8x10^{13}$vg/kg when applied to a patient. In fact, in a recent clinical trial for hemophilia using a liver-directed approach with AAV vectors, the maximal dose used was $2x10^{12}$ vg/kg and these patients developed an immune response triggered by the vector (Manno *et al.*, 2006).

Our data suggest that AAV8 does not have a preferential tropism for the pancreas when delivered systemically (intravascular o intraperitoneal), and "saturating" doses are required to allow the vector to target the pancreas. Instead, when AAV8 is

injected locally into the pancreas via the ducts, high transduction is achieved by using moderate doses of the vector, indicating that the limiting step in pancreas transduction is not the entry of the virus into the pancreatic cells, but rather targeting the pancreatic tissue.

Although we clamped the liver at the time of virus injection, in addition to pancreas transduction we also observed liver transduction. Similar results have been reported by other groups when using this approach (Loiler *et al.*, 2005; Wang *et al.*, 2006). This unexpected transduction has to be taken into account when the expression of the gene of interest is controlled by a ubiquitous promoter. To prevent expression of the transgene in undesired tissues, cell-type specific promoters can be used to direct the gene expression to ß-cells or acinar cells. In this study we have examined this possibility by using adenoviral vectors expressing the ß-gal gene under the control of the rat insulin promoter-II. Systemic injection of these vectors in mice with clamped circulation limited the expression of the ß-galactosidase marker to ß-cell, with no expression detectable in the liver or the exocrine tissue. In addition, the use of tissue-specific promoters could be advantageous since it has been demonstrated that this approach reduces the immune response against the transgene when adenoviral vectors are used (Pastore *et al.*, 1999).

Several studies have shown that ß-cell precursors reside both in pancreatic ducts and inside mouse islets (Rosenberg, 1995; Weir and Bonner-Weir, 2004; Trucco, 2005). These cells may be able to participate in islet regeneration and/or survival given the appropriate stimuli. Therefore, synthesis and release of (soluble) factors involved in pancreas regeneration (George *et al.*, 2002), ß-cell differentiation (Kojima *et al.*, 2003), and/or ß-cell preservation and function (Giannoukakis *et al.*, 1999b; Grey *et al.*, 1999;

Heimberg *et al.*, 2001) might be possible from murine and canine pancreatic islets or ducts *in vivo* by applying the techniques described in this work. In order to design a new gene therapy approach for this disease, here we have generated and tested both HD-Ad and AAV8 vectors carrying the IGF-I gene and new experiments to transfer this gene to the pancreas of diabetic mice are ongoing. The capacity of this therapy to regenerate the endocrine pancreas and revert the disease will be examined. In addition to delivering potential therapeutic genes to treat diabetes, the methodologies described herein may also be used to study islet biology and to develop new therapeutic strategies for other pancreatic disorders.

V. CONCLUSIONS

PART I

1. Bone marrow cells from ß-actin/GFP transgenic donor mice were transplanted into IGF-I transgenic mice and high chimerism in peripheral blood cells was observed one month after the transplant, indicating that bone marrow transplantation was successful.

2. Transgenic mice developed marked hyperglycemia, but gradually decreased blood glucose, suggesting that ß-cell regeneration occurred in these mice. Pancreatic islets from these transgenic mice presented altered distribution of α and ß cells after STZ-treatment, suggesting that few ß-cells were destroyed and regenerated.

3. Only 5 GFP+/Insulin+ cells in total were detected after the analysis of pancreas tissue from 32 transplanted mice, suggesting that bone marrow cells do not participate in the maintenance of the β cell mass in adults. In addition, neither IGF-I overexpression nor the STZ-treatment increased the recruitment of bone marrow cells into the islets.

4. Bone marrow-derived cells in the interstitium of the exocrine and endocrine pancreas expressed the panhematopoietic marker CD45, suggesting that transdifferentiation did not occur.

5. Groups of GFP expressing cells were found surrounding blood vessels, however, there was no evidence supporting that these cells were incorporated into the vessel wall.

6. The data suggests that replication of pre-existing ß-cells and/or differentiation from non-BMC precursors is the most likely mechanism for IGF-I-mediated islet regeneration.

PART II

1. High concentrations of adenoviral vectors can be maintained in the bloodstream of mice by clamping the hepatic circulation and this technique allows transduction of murine pancreas *in vivo.*

2. Seven days after vector administration about 70% of pancreatic islets showed ß-gal expression, with an average of about 20% of the cells within positive islets being transduced. However, this percentage decreased at 14 days and almost disappeared 30 days after the administration.

3. About 0.3% of scattered acinar cells expressed the marker gene seven days after vector injection and this percentage was almost maintained after 30 days.

4. The utilization of the rat insulin promoter avoids expression in the liver and exocrine pancreas and allowed high expression of the marker gene in ß cells.

5. Gene transfer to pancreatic islets was achieved by systemic injection of helper-dependent adenoviral vectors in mice with clamped hepatic circulation.

6. Efficient gene delivery to the exocrine and endocrine pancreas was detected after intraductal administration of AAV8 vectors. Intravenous or intraperitoneal injection of AAV8 resulted in liver, but not pancreatic, transduction, even when the portal clamp technique was used.

7. Helper-dependent adenovirus and AAV8 vectors expressing IGF-I have been generated and *in vivo* pancreatic transduction in mice and IGF-I expression in islet cells was observed.

8. *In vivo* gene transfer to healthy canine pancreas was possible by adenovirus injection into the pancreaticoduodenal vessels in beagle dogs in which pancreatic circulation was clamped.

9. ß-gal was expressed mainly in acinar cells, but also in ducts and islets. Moreover, transduction was prominent in connective tissue of the lobe septa.

10. Surgery and vector administration was feasible in a diabetic dog and ß-gal expression in the exocrine pancreas was similar to that of healthy dogs.

11. The techniques described herein may be used to study islet biology and also to assay new gene therapy approaches for diabetes mellitus and other pancreatic disorders.

VI. MATERIAL AND METHODS

MATERIALS and METHODS

1. ANIMALS

Male and female transgenic mice expressing the chimeric gene RIP/IGF-I with a C57Bl6/SJL genetic background (George *et al.*, 2002) were used as a recipient for bone marrow transplantation. Male transgenic mice expressing GFP under the ubiquitous ß-actin promoter with a C57Bl6 genetic background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and were crossed with SJL mice to obtain GFP transgenic mice with hybrid C57Bl6/SJL background. Six week old F1 generation were used as bone marrow donors. Male and female control and IGF-I transgenic mice aged 2 months were used as recipients. Male C57BL/6SJL mice, 2-month old, were used (CBATEG) for comparison of pancreatic islet size and number.

For gene transfer sudies, 8-12 week old male CD-1 mice (CBATEG) were used in all experiments. Mice were fed *ad libitum* with a standard diet (Panlab) and kept under a light-dark cycle of 12 h (lights on at 8:00 a.m.).

Male Beagle dogs, 6-12-months old, were used (Isoquimen). They were fed once a day with a standard diet and kept under a natural light cycle.

Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

2. BONE MARROW TRANSPLANTATION.

Bone marrow from male donor mice was flushed from the medullary cavities of tibiae and femurs with RPMI medium using a 25-gauge needle under sterile conditions.

Marrow cells were checked for viability with the trypan blue method and filtered. Recipients were irradiated using a myeloablative regimen (10 Gy fractionated in two doses, 4 h apart) with a Philips MG324 X-ray equipment (Philips) set at 300 kV, 10 mA, delivered a dose rate of 1.03 Gy/min. Recipients received approximately 10^7 unfractionated bone marrow–derived cells by tail vein injection. Recipient mice were kept in sterile cages with sterile chow and water for 2 weeks after irradiation. Because all animals had the same background, no alloimmune or graft-versus-host response was expected (nor observed). Chimerism was determined in peripheral blood nucleated cells by FACS analysis (FACSCalibur, BD) and was expressed as the percentage of GFP positive cells.

3. DIABETES INDUCTION.

One month after BM transplantation, mice were given, on 5 consecutive days, an intraperitoneal injection of STZ (35mg/kg), dissolved in 0.1M citrate buffer (pH 4.5) immediately before administration. Thirty-five days after the first STZ treatment these mice were injected a second time with 5 consecutive injections of STZ (40mg/kg). Control mice were injected only with the citrate buffer.

Experimental diabetes was induced in one dog by a single intravenous injection (by cephalic vein) of a mixture of streptozotocin (STZ) (35mg/kg body weight) and alloxan (40mg/kg body weight)(Anderson *et al.*, 1993).

4. DNA BASIC TECHNIQUES.

4.1 Bacterial culture.

We have used XL2-blue (Stratagene), XL10-Gold (Stratagene), BJ 5183 (Transgene) and DH5α (Invitrogen) bacterial strains cultured in LB medium (Conda) in the presence of the appropriate antibiotic. When cells were grown on a solid medium, 2% of agar was added to the LB medium.

4.2. Plasmid DNA preparation.

Minipreparations of plasmid DNA were performed using the alkaline lysis protocol originally described by Birnboim and Doly (1979).

When higher amounts of plasmid DNA were needed, the *QIAGEN Plasmid Maxi kit* (Qiagen) was used. In this case up to 500 μg DNA could be obtained.

4.3 DNA enzymatic modifications.

4.3.1 DNA digestion with restriction enzymes.

Each restriction enzyme required specific reaction conditions of pH, ionic strength and temperature. Therefore, in each case, the manufacturer's instructions were followed (New England Biolabs, Roche or Promega). In general, DNA was digested at a concentration of 0.5μg/μl using 1-4 units of the enzyme per μg of DNA. Digestions were carried out for 2-3 h in the specific buffer and the digestion products were analyzed in agarose gels. When DNA was to be cleaved with two or more restriction enzymes, the digestions could be carried out simultaneously if both enzymes worked well at the same temperature and in the same buffer. If the enzymes had different

requirements, after the first digestion the DNA was purified from salts and enzyme etc using the *QIAEX II® Gel Extraction kit* (Qiagen) according to the manufacturers instructions. The DNA was eluted in the desired volume of water and the second digestion performed directly.

4.3.2 Dephosphorylation of DNA fragments.

DNA can be rendered resistant to self-ligation by enzymatic removal of phosphate residues from their 5' termini with phosphatases. The *Shrimp Alkaline Phosphatase* (SAP) (Promega) was used and dephosphorylation reactions were performed for 30 min at 37ºC with the manufacturer's buffer. Upon completion, the enzyme was inactivated by heating to 65ºC for 15 min.

4.3.3 Generation of blunt ends in DNA fragments.

The filling-in of 5'-protruding ends or the shortening of 3'-protruding ends allows the cloning by blunt-end ligation of non-compatible restriction sites. The enzyme used for blunt end generation was the *Klenow DNA polymerase I fragment (*New England Biolabs*)*. In the presence of double-stranded DNA and deoxynucleoside triphosphates (dNTPs), this enzyme fills the gaps left by restriction enzymes that produce 5' protruding ends. In the absence of dNTPs the enzyme eliminates the 3' protruding end nucleosides. The reaction was performed following the manufacturer's instructions.

4.3.4 Ligation of DNA fragments.

The bacteriophage *T4 DNA ligase* was used for the ligation reactions following the manufacturer's instructions (New England Biolabs). The reaction was carried out in the presence of the ligation buffer with ATP for 2-3 h at 16ºC, in the case of cohesive ends fragments, and at 18ºC o/n, in the case of blunt end fragments.

4.4 DNA resolution and purification.

Electrophoresis through agarose gels was the standard method used to separate, identify and purify DNA fragments. One per cent agarose gels were used to resolve DNA fragments between 0.5-7 kbp. The location and relative size of DNA within the gel was determined by staining the gel with low concentrations of the fluorescent dye ethidium bromide which intercalates between the two strands of the DNA. The presence of the DNA was visualized with low wavelength (310 nm) ultraviolet (UV) light using the transilluminator and camera system (Syngene). The following DNA size markers were used: Marker X (Roche) or DNA ladder 1 kb (Invitrogen).

Gels were prepared by dissolving agarose in 1x TAE (Tris-acetate pH 8.3, 40mM and EDTA 1mM) electrophoresis buffer containing 0.5µg/ml ethidium bromide. Samples were loaded in 1x loading buffer and electrophoresesed in 1x TAE electrophoresis buffer at 75 V.

To extract and purify a DNA fragment from the agarose gel the *QIAEX II Gel Extraction kit* (Qiagen) was used. DNA was quantified in a spectrophotometer measuring the absorbance at 260 and 280nm in 1ml quartz cuvettes.

4.5. Transformation of competent *E. coli.*

Plasmid DNA can be introduced into competent bacteria by the process of transformation which involves either heat-shock or electroporation.

A) Heat-shock transformation

Competent cells were thawed on ice at the moment of use and 1-3µl (1-10ng) of the DNA ligation reaction or control DNA was added directly to the cells. Cells and

DNA were mixed and incubated on ice for 30 min. After that, a heat-shock of 1 min in a 42ºC water bath was applied after which cells were immeditaely put on ice for 2 min. Nine hundred µl of LB was added and cells were incubated for 1 h at 37ºC with moderate shaking. Following this, 100 µl of the suspension was plated in LB plates with the appropriate antibiotic and incubated at 37ºC o/n.

B) Electrotransformation

In this technique, a high-voltage electric field was applied briefly to cells, apparently producing transient holes in the cell membrane through which plasmid DNA enters. One µl (about 10ng) of the DNA ligation reaction or control DNA was added to 40µl of recently thawed competent cells, mixed and kept for 1 min on ice. The cell mixture was then placed in a chilled cuvette and electroporated in a BIORAD machine. Immediately after the electroporation, 1ml of LB was added to the cuvette, mixed thoroughly and incubated at 37ºC for 1 h with moderate shaking. Cells were plated as described above (section 4.5A) for the heat-shock transformation.

4.6. Construction of plasmids to generate viral vectors.

4.6.1.Construction of pKP1.4 RIP-II/ßgal.

To generate the E1 deleted adenoviral vector carrying the ß-gal gene under the control of rat insulin promoter-II (RIP-II), we used the shuttle plasmid PTG600 (Genethon). PTG600 contains two homologous regions with Ad5 genome flanking the expression cassette and the pKP1.4Δ plasmid (Genethon) which is the E1deleted Ad5 genome inserted in a bacterial backbone. The pSP72 plasmid with the RIP-II was kindly provided by M. Magnuson (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA). The RIP-II

promoter was digested from pSP72 with *BamHI* and *HindIII* and was subloned in the PTG6600 in the *BglII – HindIII* sites (which removed the CMV promoter) by ligation with T4 ligase (New England Biolabs). The resulting plasmid was named PTG6600 RIP-II. Next, the ß-gal gene was obtained by *NotI* digestion of the pCMVß plasmid (Clontech) and inserted in the *NotI* site of the multiple cloning site in the PTG6600 RIP-II plasmid. The resulting plasmid was named PTG6600 RIP-II/ß-gal. This plasmid was digested with *BstEII* and pKP1.4Δ was digested with *SwaI* and the two were subsequently co-transformed in a especial bacterial background with high efficiency of recombination *E. coli* BJ 5183 *recBC sbcBC* (Transgene) and incubated o/n 37ºC. The following day, plasmid was isolated from BJ bacterial colonies and was transformed in Xl2-blue bacteria (Stratagene) to avoid further recombination, since these bacteria have a genetic background with mutated genes for recombination (*recA1*-). After plasmid isolation from Xl2-blue bacteria, correct clones were identified by several digestions with different restriction enzymes. The plasmid containing the E1-deleted Ad5 genome containing the RIP-II/ßgal was named pKP1.4Δ RIP-II/ß-gal and was used to generate the viral particles.

4.6.2. Construction of the helper-dependent adenoviral vector expressing IGF-I (pEA0).

The 0.8kb *EcoRI-EcoRI* fragment of murine IGF-I cDNA was cloned in the *EcoRI* site of the PTG6600 plasmid, resulting in the PTG6600 CMV/IGF-I plasmid. Then, the entire expression cassette containing the CMV promoter, IGF-I cDNA and the polyA sequence was excised with *BglII* and *MfeI* and was blunt-ended (section 4.3.3).

In parallel, the shuttle vector to generate HD-Ads, pSTK129, was digested with *SwaI*, generating blunt ends. pSTK129 contained the ITRs, the packaging signal and

stuffer DNA from HPRT (Edwards *et al.*, 1990) and C346 (Andersson *et al.*, 1995). The ligation of the *SwaI*-digested vector and the CMV/IGF-I cassette resulted in the pEA0 plasmid, which was the HD-Ad plasmid carrying the IGF-I used to generate viral particles.

4.6.3. Construction of AAV RIP-II/GFP.

The cDNA of eGFP was digested with *NheI* and *Asp718I* from the eGFP-c1 plasmid (Clontech) and was subcloned into the same restriction sites of PTG6600 RIP-II to generate the PTG6600 RIP-II/GFP. Thereafter, the RIP-II/GFP expression cassette was removed by digestion with *SgrAI* and *MfeI* and was blunt-ended (section 4.3.3). The AAV backbone plasmid pAAV-MCS (Stratagene), containg the essential ITRs, was digested by *NotI* (eliminating the CMV promoter and MCS) and was blunt-ended using the Klenow enzyme. The RIP-II/GFP expression cassette was then cloned into the AAV plasmid and the resulting plasmid containing the RIP-II/GFP cassette flanked by the AAV ITRs was named AAV RIP-II/GFP, and was used to generate the viral particles.

The AAV-CAG-GFP-WPRE was generated previously in our laboratory and contained the CMV enhancer/chicken ß-actin hybrid promoter, the eGFP cDNA, and the WPRE sequence to increase RNA stabilization (Figure 1).

The AAV-CAG-IGF-I-WPRE was generated previously in our laboratory and contains the CMV enhancer/chicken ß-actin hybrid promoter, the murine IGF-I cDNA, and the WPRE sequence to increase RNA stabilization (see Results Section ***2. 1.2***).

The AAV-CMV/βgal plasmid was obtained from M. Giacca (ICGEB, Trieste, Italy) (Figure 2).

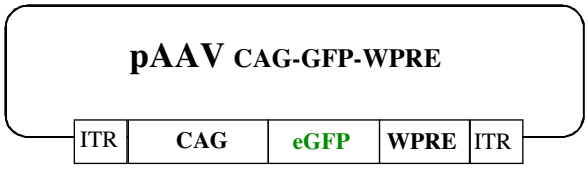


Figure 1. Schematic representation of pAAV-CAG-GFP-WPRE vector. ITR=inverted terminal repeats. WPRE=Woodchuck Posttranscriptional Regulatory Element, CAG= Chiken β-actin Promoter.

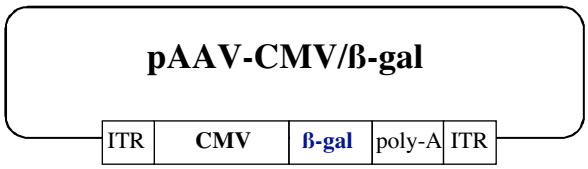


Figure 2. Schematic representation of pAAV-CMV/β-gal vector. ITR=inverted terminal repeats, CMV= Cytomegalovirus Promoter, poly-A= polyadenilation signal.

5. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF FIRST-GENERATION ADENOVIRAL VECTORS

Human E1-deleted recombinant serotype 2 adenoviruses carrying the cytomegalovirus promoter/ß-galactosidase (AdCMV/ß-gal) or eGFP (AdCMV/GFP) chimeric gene were generated using the method described by Anderson *et al.* (2000) (Anderson *et al.*, 2000). The encoded ß-galactosidase protein contains a nuclear localization signal.

To generate new adenoviral vectors carrying the ß-galactosidase gene under the control of rat insulin promoter-II, the pKP1.4 RIP-II/ß-gal plasmid was first digested with *PacI*. This digestion removed the bacterial sequences of the construct and released the linear Ad genome. The released Ad DNA (3 μg) was transfected into HEK 293 cells cultured in one well of a 6-well plate, to generate viral particles. Seventy-two hours later, culture medium and cells were harvested and freeze/thawed 3 times in liquid N_2

and a water bath at 37ºC. Cell debris were removed by centrifugation at 3,000 rpm for 10 min. This supernatant was used to infect one 10 cm Petri dish of HEK 293. Cells and medium were collected 72 h after, and were frozen/thawed 3 times. Cell debris were removed by centrifugation at 3,000 rpm for 10 min and the supernatant was used to infect one 15 cm petri dish. Cells and culture medium were harvested 36 h after the infection and were freeze/thawed 3 times and centrifuged as described above. This supernatant was used to infect twenty 15 cm dishes. After 36 h, cells and medium were harvested and centrifuged at 1000 rpm for 5 min. Most of the culture medium was discarded, leaving only 30 ml with the cell pellet. This solution was freeze/thawed 3 times and centrifuged at 3000 rpm for 10 min. Adenoviral vectors were purified from this supernatant by two CsCl gradient ultracentrifugation steps. The first step consisted of a discontinuous gradient using 1.27 g/ml and 1.41 g/ml (bottom) CsCl density in an ultraclear tube (Beckman). Without mixing, the supernatant containing the virus was loaded on top and the solution was ultracentrifuged at 35,000 rpm for 2 h at 18ºC in a Sw40Ti rotor (Beckman). After centrifugation, a white adenovirus ring appears. The virus layer, about 2 ml, was collected carefully using a syringe and mixed with 10 ml of CsCl solution with a density of 1.34. Tubes were ultracentrifuged at 35,000 rpm for 19-21 h (o/n) at 18ºC in a Sw40Ti rotor. A white adenovirus ring of about 1-2 ml was collected using a syringe and was loaded onto a Sephadex column (Amersham PD-10) which had been previously equilibrated with 25 ml PBS. When the virus solution had completely entered the column, 5 ml of PBS was added on top of the column and the eluted solution was collected in 1.5 ml tubes in 5 fractions (1000 µl each). Second and third fractions were pooled and stored in 10% glycerol at -80ºC.

The physical particles were measured by spectrophotometry at 260 nm. Infection units (IU) were determined by infecting HEK 293 cells with serial dilutions of

the virus and then counting ß-gal or GFP expressing cells after 48 h. For titration of the RIP-II/ß-gal adenoviral vectors, this method cannot be used since the rat insulin promoter is not active in HEK 293 cells. Thus, titration was performed using the *Adeno-X™ Rapid Titer kit* (Clontech). This method takes advantage of production of viral hexon proteins for the quantification of viral stocks. Dilutions of the viral stock were used to infect HEK 293 cells. Forty-eight hours later, these cells were fixed and stained with the antibody specific to the adenovirus hexon protein from the kit. Signal was detected using a secondary antibody conjugated with horseradish peroxidase (HRP) to amplify the signal of the anti-hexon antibody. Subsequent exposure to metal-enhanced 3,3'-diaminobenzidine (DAB) peroxidase substrate stained only the infected cells dark brown. The titer of the stock could then be determined by counting the number of brown cells in a given area. Each stained cell corresponded to a single IU. The particle/IU ratio of the virus stocks used in the experiments was between 10-40.

6. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF HELPER-DEPENDENT ADENOVIRAL VECTORS

6.1 Production and purification

In collaboration with Stefan Kochanek and Florian Kreppel (Ulm University, Ulm, Germany) we have stablished the production of HD-Ad vectors in our laboratory. To generate HD-Ad vectors expressing marker genes, pGS46 (HD-Ad CMV/ß-gal), pFK7 (HD-Ad CMV/GFP), the GS102 helper virus and the cell line Cre66 were used. All this

material was obtained from S. Kochanek Laboratory. Schematic representation of these plasmids is shown in Results section.

The helper virus (HV) was amplified in ten 15 cm dishes and was purified in CsCl as described for first-generation adenoviral vectors (section 5). To calculate the optimal cytopathic effect (CPE) in Cre66 cells, we infected 6cm dishes ($2x10^6$ cells) with different amounts of the HV stock (10^{11} pp/ml) from 0.1 µl to 10 µl. We found that 1 µl induced the desired CPE effect. That is, 50% of the cells remained attached to the dish and 50% were observed to detach, and all cells had a round shape.

To generate the HD-Ad, the bacterial backbone was released from the shuttle plasmids (pGS46, pFK7 or pEA0) by digestion with *Pme*I. The linearized shuttle plasmids were transfected into $2x10^6$ HEK 293-Cre66 cells by calcium phosphate co-precipitation. Two hours after transfection, the HEK 293-Cre66 cells were infected with helper virus AdGS102 (using the optimal CPE). The cells were harvested 48 h after infection, were freeze/thawed 3 times and were centrifuged at 3000rpm for 10 min. The supernatant was used for serial amplifications in HEK 293-cre66 cells. The second amplification was carried out in one 6cm dish ($2x10^6$) of Cre66 cells infected with the HV and 2 h later with the supernatant from the first amplification. The third amplification was performed in one 6 cm dish using the same procedure while the fourth amplification was carried out in one 15 cm dish using the supernatant of the 3rd amplification. The fifth amplification was carried out in two 15 cm dishes. Ten 15 cm dishes (2x107 Cre66 cells per dish) were infected with the fifth amplification supernatant, Cells were harvested 48 h later and were freeze/thawed 3 times and the lysate purified by double CsCl gradient ultracentrifugation as described for FG-Ad vectors (section 5) and desalted using PD-10 columns (Amersham). The physical particle titers were determined by the slot blot procedure (section 6.2) and the integrity

of the vector genomes was confirmed by restriction digestion. Helper virus contamination was also determined by the slot blot procedure. After purification and characterization of these virus stocks (HD-Ad CMV/ßgal, HD-Ad CMV/GFP or HD-Ad CMV/IGF-I), we used them to infect twenty 15 cm dishes of Cre66 at a multiplicity of infection (MOI) of 20. After purification and titration of these vectors we obtained $4.5x10^{11}$pp/ml (0.2% HV contamination) of HD-Ad CMV/GFP, $1x10^{12}$ pp/ml (2.4% HV contamination) of HD-Ad CMV/ß-gal and these preps were used for the *in vivo* experiments. The HD-Ad CMV/IGF-I had a titer of $2x10^{11}$ pp/ml (1.1% HV contamination).

6.2 Slot blot titration

The slot blot procedure used to titer the HD-Ad vectors was based on the protocol described by Kreppel *et al.* (2002) (Kreppel *et al.*, 2002a). The viral stock was diluted (1/20 or 1/200) or used undiluted. The different amounts of vector and the standard curves, made using Hd-Ad plasmid or HV plasmid, were added to 1.5 ml tubes and combined with 200 µl PBS/EDTA (50 mM). Afterwards, 200 µl of 0.8N NaOH was added to each tube, mixed thoroughly and incubated at room temperature for 30 min. This solution was transferred to a nylon membrane (Hybond, Amersham), previously soaked with 0.4N NaOH, using a slot blot apparatus (Amersham). After the transfer, the membrane was washed twice in 2% SSC (15 mM NaCl, 1.5 mM Na-Citrate, pH 7.0) and the DNA was covalent linked to the membrane by irradiation with 120000 µJ of radiation during 25-50 seconds, using the UV-Stratalinker 1800 (Stratagene).

6.2.1. Radioactive labelling of DNA probes.

For HD-Ad vectors we used a DNA probe containing a fragment of the HPRT gene and for HV virus we used a DNA probe containing part of the Ad5 fiber gene. These DNA probes were labelled with [α-^{32}P]-dCTP (3000 Ci/mmol; Amersham) using the commertial kit *Ready-To-Go® DNA labelling Beads* (Amersham) following manufacturer's instructions. After radiactive labelling, the DNA probes were filtered using *Sephadex G-50* (Probe Quant® G-50 Micro Columns, Amersham) to discard unincoporated radioactive nucleotide that could increase the background signal.

6.2.2. Prehybridisation, hybridization and quantification.

Nylon membranes were prehybrized at 65ºC for 2 hr in 20 ml prehybridisation buffer (Na_2HPO_4 0.25 mM pH 7.2, SDS 20%, EDTA 1 mM containing 0.5% Blocking Reagent (Roche) Radioactive probes were added to the tubes and incubated o/n at 65ºC with continuous rotation.

The membranes were washed twice with prewarmed Wash Buffer I (2xSSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) and 0.1 % SDS) for 10 min at 68ºC, then washed once with prewarmed Wash Buffer II (0.1xSSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) and 0.1 % SDS) for 10 min at 68ºC and once again with wash buffer II for 5 min at room temperature. Then, the membranes were exposed in PhosphorScreen casettes (Fujifilm FLA-3000) and quantified using the software Image Gauge v4.0 (Fujifilm). We calculated signal intensities of the HD and HV plasmid standard curves and used linear regression to quantify the vector samples. The quantification was considered valid only when the correlation for the standard curve was above 0.99.

7. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF AAV VECTORS.

7.1 Production and purification.

To generate the AAV vectors expressing marker genes, we used the previously constructed AAV-CAG-GFP-WPRE and AAV-CMV-ßgal plasmids. For selective expression of the marker genes in ß-cells we constructed the AAV-RIP-II/GFP as described above. Schematic representation of these plasmids is shown in Results section.

These vectors were generated in collaboration with M. Giacca lab in the International Centre for Genetic Engineering and Biotecnology (ICGEB), Trieste, Italy. Infectious AAV8 vector particles were generated in HEK 293 cells cultured in 150 mm-diameter Petri dishes, by co-transfecting each plate with 15 µg of the vector plasmid (containing the ITRs and the expression cassette; pCAG-GFP-WPRE, pCMV/ßgal, pCAG-IGF-I-GFP, or pRIP-II/GFP) together with 15 µg of the rep/cap plasmid expressing the AAV proteins of the AAV8 particle (pAAV8; kindly provided by K. High, Children's Hospital, Philadelphia, USA), and 30 µg of the helper plasmid pWEAD expressing adenovirus helper functions (kindly provided by K. High) by calcium phosphate co-precipitation. A total of 30 plates were used for each vector preparation. Cells were harvested, pelleted at 1000 rpm for 20 min at 4ºC, and resuspended in 50 mM HEPES pH 7.6, 150 mM NaCl. After 3 cycles of freeze/thaw the lysate was centrifuged at 3000 rpm for 20 min. The supernatant was conserved in a new tube and the pellet resuspended again with 50 mM HEPES pH 7.6, 150 mM NaCl and freeze/thawed 3 times. The second supernatant was mixed with the first one, and 1/3 vol of cold saturated ammonium sulfate was added. Tubes were incubated on ice for

10 min and then centrifuged at 5,000 rpm at 4ºC for 10 min. The pellet was discarded and 2/3 vol of cold ammonium sulfate was added to the supernatant, incubated for 20 min on ice and then centrifuged at 12,000rpm at 4ºC for 20 min. The supernatant was discard and the pellet resuspended in 20 ml of CsCl 1.37 g/ml density. This solution was divided between two ultra clear tubes (Beckman) containing 0.5 ml of CsCl 1.5 g/ml density at the bottom and were centrifuged at 39,000 rpm at 18ºC for 36-48 h in a SW41 rotor (Beckman). After centrifugation, one hole was made near the bottom of the tube using a syringe and 0.5 ml fractions were collected and were measured using a refractometer. Fractions with a refractometer measure between 1.385-1.369 were pooled and dialyzed using 10000 Da membrane (Slide-A-Lyzer Dialysis Products, Pierce). AAV titers were determined by measuring the copy number of vector genomes using radioactive slot blot (section 7.2). Viral preparations used in this work for animal transduction had titers between $1.5x10^{11}$ and $7x10^{11}$ viral genomes per ml.

7.2 Slot blot titration of AAV vectors.

7.2.1. Viral DNA extraction by proteinase K digestion.

Two µl of the vector stock was digested in a solution containing 40 ul of proteinase K solution (100 mM Tris-HCL, pH 8.0; 0.2 mM EDTA;1mg/ml proteinase K (Roche); 1% SDS) and 38 µl of sterile H_2O. This solution was mixed by vortexing and incubated at 37ºC for 1h. Afterwards, the solution was cooled to room temperature. One hundred and twenty µl of sterile H_2O and 200 µl phenol-chloroform-isoamyl alcohol (25:24:1), was added and then was centrifuged at 13000 rpm 10 min 4ºC. The upper phase was taken and was transferred into a new tube with 200 µl chloroform-isoamyl alcohol (24:1) and the solution was centrifuged 13000 rpm 10 min 4ºC. The upper phase was taken and transferred into a new tube with 2 µl glycogen (20 mg/ml, Roche), 20 µl

of 3 M sodium acetate (pH 5.2), and 450 µl ethanol. To facilitate precipitation the sample was incubated at -80^{o}C for 1 h. Afterwards, the tube was spinned at maximum speed for 20 min at 4ºC, and the supernatant was removed carefully. The pellet was resuspended in 200 µl cold 70% ethanol and centrifugated 13000 rpm for 5 min. The supernatant was removed carefully and the pellet was air-dried for 10 min. Finally the pellet was resuspend in 10 µl of TE buffer. This solution containing the viral DNA was divided in four tubes (5 µl, 2.5 µl, 1 µl and 0.5 µl, respectively) containing 150 µl of spotting solution (0.4 N NaOH, 25 mM EDTA, 0.00008% Bromothymol Blue) each. In addition, to prepare the standard curve serial dilutions of plasmid standards (5 µl each) were mixed with spotting solution (150 µl each). The plasmid standards used were 10 ng, 5 ng, 2.5 ng, 1 ng, 0.5 ng, 0.05 ng, 0.01 ng (each in 5 µl).

7.2.2. Transfer of DNA to the membrane, hybridization and quantification.

The 150µl spotting solution containing the standard curve DNA or viral DNA was transferred to a nylon membrane (Hypobond, Amersham) wetted with pre-wetting solution (0.4 N NaOH, 25 mM EDTA) using a slot blot apparatus (Amersham). After the transfer, the membrane was washed twice in 2% SSC and the DNA was covalent linked to the membrane by irradiation with 120000 µJ of UV light during 25-50 seconds, using the UV-Stratalinker 1800 (Stratagene). Radioactive hybridization and quantification of the DNA was done as already described for HD-Ad vectors (section 6.2.2).

8. RNA ANALYSIS.

Total RNA was obtained from *tibialis cranialis* muscles or A549 cells following manufacture's instructions (TriPure Isolation Reagent, Roche) and RNA samples (20µg) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to nylon membranes (Roche) and membranes were hybridized to an ^{32}P-labeled 0.8-kb *EcoRI-EcoRI* fragment containing the entire IGF-I cDNA or to a 150 bp fragment, synthetized by PCR (using the following primers; primer 5' ATTCGCTGCACGAACTGCG and primer 3' CAGCAGGTCTGAATCGTGGT), containing the partial sequence of murine ribosomal protein S26 (rbs) as a housekeeping gene. The hybridization, washing and exposure of the Northern blot were performed as described in the slot blot protocol (section 6.2). Equal loading was also documented using a picture of the membrane containing the RNA stained with methilene blue, in this case the 18s band was shown.

9. *IN VIVO* ADMINISTRATION OF VIRAL VECTORS IN MICE.

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). For the liver clamp, a 3-4 cm ventral midline incision was made starting just caudal to the xyphoid process and continued caudally. The abdomen was entered through another ventral midline incision through the *linea alba*. Following the laparatomy, the hepatic triad (portal vein, hepatic artery and bile duct) was clamped (Ye *et al.*, 2000). Afterwards, 100 µl of virus suspension (the proper amount of virus stock dependending on the dose and up to 100 µl with saline solution)

was injected into the jugular vein. Thirty minutes later, the clamp was removed and the abdomen was rinsed with sterile saline solution and closed with a two-layer approach.

For intraductal delivery, mice were anesthetized and the abdominal wall was opened as described above. The duodenum was isolated with the common bile duct attached. A syringe with a 30-gauge needle was used to make an opening in the duodenum and advanced retrogradely through the sphincter of Oddi into the common bile duct. The needle was secured in place with a microclamp around the bile duct to avoid leakage of the vector to the intestine. Another microclamp was placed on the bile duct just caudal to the liver to prevent vector distribution to the liver. The AAV vector was slowly injected into the pancreatic duct with PBS in a total volume of 100 µl. After approximately 1 minunte the microclamps and the needle were removed and the abdomen rinsed with sterile saline solution and closed with a two-layer approach.
For intraperitoneal injection, 100 µl of virus suspension was used.

10. LOCAL PANCREATIC CLAMP AND ADMINISTRATION OF ADENOVIRUS IN DOGS

All dogs received an IM injection of neuroleptoanalgesic combination of acepromacine (0.05 mg/kg) and buprenorfine (0.01 mg/kg). Thirty minutes after the preanesthetic medication, anesthesia was induced by intravenous injection of 4mg/kg of propofol, and maintained with 1-2% of isofluorane in oxygen. Following laparatomy, pancreatic circulation was clamped (including celiac, splenic, gastroduodenal, gastroepiploic and cranial and caudal pancreaticoduodenal arteries). Blood vessels were occluded with haemostatic clamps. Since pancreas and duodenum share a common blood supply, we also clamped the duodenum by plastic protected intestinal clamps.

Afterwards, 2 ml of virus suspension (in NaCl 150 mM) was injected into the pancreaticoduodenal vein (or artery). A total viral dose of $2x10^{10}$ IU per animal was used. Ten minutes later, the clamp was removed and the abdominal wall was sutured. Surgery and vector dose (injected in the pancreaticoduodenal vein) used in unclamped dog was the same as in clamped dogs, however, blood vessels were not occluded. Five days after vector administration, animals were euthanasied with an overdose pentobarbital injected intravenously and the pancreas and liver removed.

11. MEASUREMENT OF ADENOVIRUS CONCENTRATION IN BLOOD

Serum samples were taken 5, 30, 35 and 60 min after AdCMV/ß-gal ($5x10^8$ IU/mice) vector injection into the jugular vein. The portal circulation was clamped immediately before virus injection and this clamp was released 30 min later. We used 1 µl of the serum to infect HEK 293 cells. Since the vector used in this experiment expressed the ß-gal gene under the ubiquous CMV promoter, the ß-galactosidase activity in the infected cells could be quantitatively measured by luminometry. Twenty-four hours after the infection cells were lysated and ß-gal protein was measured using the Galacto-light Plus ^{TM} (TROPIX) and the LUMINOSCAN (Lab Biosystem). The values obtained were expressed as relative light units (RLU).

12. DETERMINATION OF SERUM PARAMETERS.

Blood glucose levels were measured with a Glucometer Elite analyzer (Bayer AG). Determination of serum Alanine aminotransferase (ALT), amylase and lipase levels were determined spectrophotometrically using specific kits from ABX DIAGNOSTICS and automated serum analyzer ABX PENTRA 400 (HoribaAbx).

13. ANALYSIS OF ß-GALACTOSIDASE EXPRESSION IN TISSUE SAMPLES

To detect the presence of ß-galactosidase in pancreas and liver *in toto*, tissue samples were fixed for 1 h in 4% paraformaldehyde, washed twice in PBS solution, and then incubated in X-Gal (5-bromo-4-chloro-3-ß-D-galactopyranoside) in 5 mM $K_3Fe(CN)_5$, 5mM $K_4Fe(CN)_6$, and 1mM $MgCl_2$ in PBS for 6-8 hrs in the dark at 37ºC.

14. INCIDENCE AND SEVERITY OF INSULITIS.

The incidence and severity of insulitis was analyzed in four paraffin sections per pancreas, each separated by 150 μm and stained with hematoxylin. The degree of mononuclear cell infiltration (insulitis score) was measured using the following rankings: non infiltrated; periinsulitis (mononuclear cells surrounding islets and ducts, but no infiltration of the islet architecture); moderate insulitis (mononuclear cells infiltrating < 50% of the islet architecture); severe insulitis (> 50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture).

15. IMMUNOHISTOCHEMICAL ANALYSIS

Pancreata were fixed for 12-24 h in formalin, embedded in paraffin and sectioned. For immunohistochemical detection of insulin, glucagon, IGF-I, GFP, CD45, amylase and α-SMA, pancreatic sections were incubated overnight at 4ºC with a guinea pig anti-insulin antibody (1:100; Sigma Chemical); with rabbit anti-glucagon antibody (1:4500; ICN Biomedicals Inc.); rabbit anti-IGF1 antibody (1:20; Gropep); goat anti-GFP (1:300; Abcam ab6673); rat anti-CD45 (1:30; BD Pharmingen 550539); rabbit

anti-Amylase (1:400; Sigma A-8273); and mouse anti alpha-SMA (1:300; Sigma A2547) respectively. Secondary antibodies were: TRITC-conjugated goat anti-guinea pig (1:500; Molecular probes), biotinilated donkey anti-goat (1:300; Santa Cruz sc-2042); biotinilated rabbit anti-rat (1:300; Dako E0467); biotinilated goat anti-rabbit (1:300; Molecular Probes, S-11226); biotinilated horse anti-mouse (1:300; Vector BA-200); streptavidin-conjugated Alexa 488 (Molecular Probes S-11223); or Streptavidin-conjugated Alexa 568 (Molecular Probes S-11226).

For immunohistochemical co-detection of ß-galactosidase and insulin, or GFP and insulin, each pancreas was fixed (for 12-24 h) in formalin, embedded in paraffin and sectioned. Sections were incubated o/n at 4ºC with a rabbit anti-ß-galactosidase antibody (1:900; Abcam ab616); rabbit anti-GFP (1:500; Molecular Probes); or guinea pig anti-porcine insulin antibody (1:100; DAKO Corp.). Secondary antibodies were: peroxidase-conjugated rabbit anti-guinea pig IgG (1:300; Roche); or biotinylated goat anti-rabbit antibody (1:300; Molecular Probes, S-11226). Antibodies were revealed with ABC complex (Vector), which employs DAB as the substrate chromogen. Sections were counterstained in Mayer's hematoxylin.

16. MORPHOMETRICAL ANALYSIS.

For morphometric analysis, immunohistochemical detection of ß-gal was performed in four (2-3 μm) sections of pancreas per animal, separated by 150 μm. The percentage of islet transduction was calculated by dividing positive islets by total islets of the pancreas section. We considered them as positive when at least one of the islet cells expressed ß-gal. The percentage of transduction within the islet was calculated by dividing ß-gal positive islet cells by total cells of the islet. Quantification of exocrine

pancreas transduction was carried out in one pancreas section per animal. We counted all ß-gal positive acinar cells in the section and measured the total area of the pancreas section. To determine acinar cell area, the mean cross-sectional area of 1000 individual acinar cells was measured in each section using a Nikon Eclipse E800 microscope (Nikon Corp.) connected to a video camera with a color monitor and to an image analyzer (analySIS 3.0, Soft Imaging System Corp.). The percentage of exocrine pancreas transduction was calculated by dividing the area of all ß-gal-positive cells in one section by the total area of this section. The area (mm^2) of each section were determined using the same image analyzer

17. STATISTICAL ANALYSIS.

All values are expressed as the means ± SEM. Differences between groups were compared by Student *t* test. *p* values less than 0.05 were considered statistically significant.

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VIII. ANNEX I

HUMAN GENE THERAPY 15:805–812 (August 2004)

Brief Report

In Vivo Gene Transfer to Pancreatic Beta Cells by Systemic Delivery of Adenoviral Vectors

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ABSTRACT

Type 1 diabetes results from autoimmune destruction of pancreatic beta cells. This process might be reversed by genetically engineering the endocrine pancreas *in vivo* to express factors that induce beta cell replication and neogenesis and counteract the immune response. However, the pancreas is difficult to manipulate and pancreatitis is a serious concern, which has made effective gene transfer to this organ elusive. Thus, new approaches for gene delivery to the pancreas *in vivo* are required. Here we show that pancreatic beta cells were efficiently transduced to express β-galactosidase after systemic injection of adenovirus into mice with clamped hepatic circulation. Seven days after vector administration about 70% of pancreatic islets showed β-galactosidase expression, with an average of about 20% of the cells within positive islets being transduced. In addition, scattered acinar cells expressing β-galactosidase were also observed. Thus, this approach may be used to transfer genes of interest to mouse islets and beta cells, both for the study of islet biology and gene therapy of diabetes and other pancreatic disorders.

INTRODUCTION

TYPE 1 DIABETES results from autoimmune destruction of the insulin-producing beta cells of pancreatic islets. Patients are identified after diabetes onset, when beta cell destruction is nearly complete. Although islet transplantation leads to successful insulin delivery (Shapiro *et al*., 2000), shortage of donors and potential elimination of transplanted islets by autoimmune reactions are serious limitations. Approaches using surrogate cells to deliver insulin (engineered cell lines derived from beta cells or nonbeta cells), are also being developed (Bottino *et al*., 2003). Nevertheless, these approaches will provide only replacement therapies. Recovery from type 1 diabetes requires beta cell regeneration from islet cell precursors and prevention of recurring autoimmunity. However, effective methods for gene delivery to the pancreas *in vivo* are required.

To genetically engineer the endocrine pancreas, selection of both the vector and the route of administration is key and needs to be further explored. Several vectors can be used to transfer foreign genes to pancreatic islets and to pancreatic beta cell lines *in vitro* (Gainer *et al*., 1996; Sigalla *et al*., 1997; Giannoukakis *et al*., 1999a; Leibowitz *et al*., 1999; Flotte *et al*., 2001; Mahato *et al*., 2003). Among them, adenovirus shows beta cell tropism and high transduction efficiency. Direct pancreatic injection and retrograde pancreaticobiliary duct delivery of adenovirus can transduce the exocrine pancreas, but these approaches induce severe inflammation and toxicity (Raper and DeMatteo, 1996; McClane *et al*., 1997a,b; Wang *et al*., 2004).

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Systemic delivery of adenovirus fails to infect pancreas *in vivo*, because the liver rapidly removes them from circulation (Ye *et al*., 2000). Injection of adenovirus to the blood stream after a temporary closure of the portal vein, hepatic artery, and bile duct (portal clamp) results in increased concentration of circulating virus during the clamp. This allows adenovirus to infect other organs such as intestine, kidney, and lungs (Ye *et al*., 2000). Here we show that systemic delivery of adenovirus to mice with clamped hepatic circulation leads to efficient gene transfer to pancreatic beta cells and scattered acinar cells.

MATERIALS AND METHODS

Animals

Male CD-1 mice, 8–12 weeks old, were used in all experiments. Mice were fed *ad libitum* with a standard diet (Panlab, Barcelona, Spain) and kept under a light–dark cycle of 12 hr (lights on at 8:00 A.M.). Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (Barcelona, Spain).

Recombinant adenoviral vector

Human E1-deleted recombinant serotype 2 adenoviruses carrying the cytomegalovirus promoter/β-galactosidase (AdCMV/β-Gal) or enhanced green fluorescent protein (AdCMV/GFP) chimeric gene were generated as described previously (Anderson *et al*., 2000). β-Galactosidase contains a nuclear localization signal. The particle:plaque-forming unit (PFU) ratio of the virus stock used in the experiments was 40.

Liver clamp and in vivo *administration of adenovirus*

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laparotomy, the hepatic triad (portal vein, hepatic artery, and bile duct) was clamped (Ye *et al*., 2000). Afterward, 100 μl of virus suspension (in NaCl, 150 m*M*) was injected into the jugular vein. Thirty minutes later, the clamp was removed and the abdominal wall was sutured. Animals were killed 3, 7, 14, or 30 days after vector administration and pancreas and liver were analyzed.

Immunohistochemistry and histopathology

To detect the presence of β-galactosidase in pancreas and liver *in toto*, samples were fixed for 1 hr in 4% paraformaldehyde, washed twice in phosphate-buffered saline (PBS), and then incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in 5 m*M* $K_3Fe(CN)_5$, 5 m*M* $K_4Fe(CN)_6$, and 1 m*M* $MgCl_2$ in PBS for 6–8 hr in the dark at 37°C.

For immunohistochemical detection of β-galactosidase and insulin, pancreata were fixed (for 12 to 24 hr) in formalin, embedded in paraffin, and sectioned. Sections were then incubated overnight at 4°C with a rabbit anti-β-galactosidase antibody (ab616, diluted 1:900; Abcam, Cambridge, UK), a rabbit anti-GFP antibody (diluted 1:500; Molecular Probes, Leiden, The Netherlands), or a guinea pig anti-porcine insulin antibody (diluted 1:100; DakoCytomation, Carpinteria, CA). As secondary antibodies rabbit anti-guinea pig immunoglobulin G, coupled to peroxidase (Roche Molecular Biochemicals, Mannheim, Germany), or biotinylated goat anti-rabbit antibody and avidin–biotin complex (ABC; Vector Laboratories, Burlingame, CA) was used. 3,3′-Diaminobenzidine (DAB) was used as the substrate chromogen. Sections were counterstained in Mayer's hematoxylin.

The incidence and severity of insulitis were analyzed in four paraffin sections per pancreas, separated by 150 μm, and stained with hematoxylin. The degree of mononuclear cell infiltration (insulitis score) was measured on the basis of the following rankings: noninfiltrated; periinsulitis (mononuclear cells surrounding islets and ducts, but no infiltration of the islet architecture); moderate insulitis (mononuclear cells infiltrating less than 50% of the islet architecture); and severe insulitis (more than 50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture).

Quantification of pancreas transduction

For morphometric analysis, pancreas was obtained and immunohistochemical detection of β-Gal was performed in four (2 to 3 μm) sections per animal, separated by 150 μm. The percentage of islet transduction was calculated by dividing positive islets by total islets of the pancreas section. We considered them positive when at least one of the islet cells expressed β-Gal. The percentage of transduction within the islet was calculated by dividing β-Gal-positive islet cells by total cells of the islet. Quantification of exocrine pancreas transduction was carried out in one pancreas section per animal. We counted all β-Gal-positive acinar cells in the section and measured the total area of the pancreas section. To determine acinar cell area, the mean cross-sectional area of 1000 individual acinar cells was measured in each section, using the same image analyzer. The percentage of exocrine pancreas transduction was calculated by dividing the area of all β-Gal-positive cells in one section by the total area of this section. The area (mm^2) of each section were determined with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) connected to a video camera with a color monitor and to an image analyzer (analySIS 3.0; Soft Imaging System, Lakewood, CO).

Statistical analysis

All values are expressed as means ± SEM. Differences between groups were compared by Student *t* test. *p* Values less than 0.05 were considered statistically significant.

RESULTS

Here we examined pancreas gene delivery in portal clamped mice injected via the jugular vein with adenovirus (5×10^8 PFU/mouse) carrying the β-galactosidase marker gene (AdCMV/β-Gal). As expected, 3 days after viral injection we did not detect pancreas transduction in nonclamped animals (Fig. 1A and D). In contrast, when the hepatic circulation was closed for 30 min, pancreas gene transfer was achieved, as evidenced by β-Gal expression throughout the pancreas in an *in toto* analysis (Fig. 1B). Transduced pancreas showed groups of β-Gal-expressing cells that were consistent with islets of Langerhans

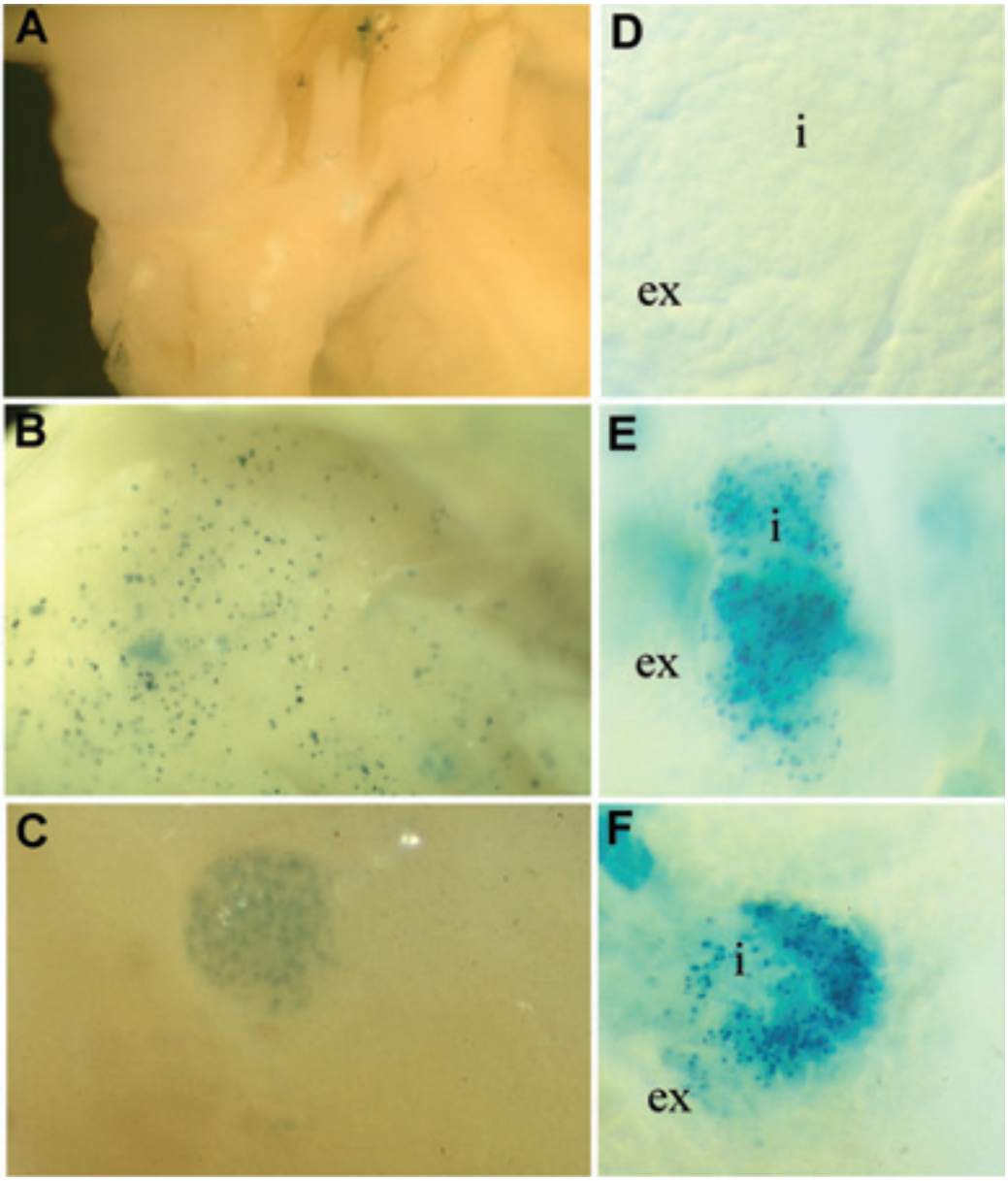


FIG. 1. Pancreas gene transfer bypassing hepatic circulation. X-Gal staining of pancreas from nonclamped (**A** and **D**) and clamped (**B**, **C**, **E**, and **F**) mice 3 days after adenovirus injection. (**A**) *In toto* analysis of pancreas showed no β-Gal expression in nonclamped animals. (**B**) Pancreas gene transfer was achieved in clamped mice. (**C**–**F**) Islets can be identified in the pancreatic parenchyma. Islets were not transduced in nonclamped animals (**D**), whereas a large number of islet cells expressing β-Gal was detected in clamped mice (**C**, **E**, and **F**). i, islets of Langerhans; ex, exocrine pancreas. Original magnification: (**A**) $\times$25; (**B**) $\times$35; (**C**–**F**) $\times$128. Representative images from nonclamped ($n = 5$) and clamped mice ($n = 8$).

(Fig. 1C, E, and F). Lower doses of adenovirus (5×10^6 and 5×10^7 PFU/mouse) and shorter clamp duration (5 and 15 min) led to inefficient pancreas transduction (data not shown). Furthermore, portal clamp longer than 30 min produces severe liver ischemia and higher viral doses induce hepatic damage (Ye *et al*., 2000).

Islet transduction was confirmed by specific β-Gal immunostaining of histological sections. Thus, 7 days after virus administration immunohistochemical analysis of pancreatic sections showed no β-Gal-positive cells in islets of animals without portal clamp (Fig. 2A). In contrast, abundant β-Gal-positive cells were observed within the islets of clamped animals (Fig. 2B and C). Distribution of transduced cells was within the core of the islet, where the insulin-producing cells reside, which agreed with beta cell tropism of adenovirus (Sigalla *et al*., 1997; Leibowitz *et al*., 1999). This was confirmed by double immunostaining for insulin and β-Gal (Fig. 2D–F). Thus, β-Gal-positive nuclei (green) were surrounded by

cytoplasmic insulin (red) (Fig. 2F), demonstrating that beta cells were efficiently transduced. Similar results were obtained when adenoviral vectors carrying GFP (AdCMV/GFP) were injected. Double immunostaining for insulin and GFP showed beta cell colocalization (Fig. 2G–I).

Morphometric analysis was performed to determine the percentage of transduced islets per pancreas 7, 14, and 30 days after adenovirus injection. This showed a high level of transduction because 71% of islets expressed β-Gal after 7 days (Fig. 2J). On day 14, about 40% of the islets were β-Gal positive. However, on day 30 a strong decrease in β-Gal-expressing islets was observed, and only two of five animals presented transduced islets (Fig. 2J). In addition, the percentage of β-Gal-expressing cells per islet was measured. Seven days after vector delivery, about 20% of islet cells were positive for β-Gal (Fig. 2K). Transduction within the islets also decreased significantly both 14 and 30 days after vector administration and only about 5 and 4% of beta cells in the positive islets, respectively, expressed β-Gal (Fig. 2K).

To evaluate the role of the immune response in the loss of β-Gal-expressing cells we measured the incidence and severity of insulitis in Ad-treated mice. We found that only a small percentage (less than 20%) of β-Gal-expressing islets presented periinsulitis, which did not progress to severe insulitis 1 month after Ad injection (Fig. 3A and B). Furthermore, mice remained normoglycemic 1 month after viral injection (nonclamped mice, 130 ± 8 mg/dl versus clamped mice, 122 ± 9 mg/dl). This indicated that pancreatic beta cell function was not altered.

In addition to beta cells, β-Gal expression was also noted in exocrine pancreas (Figs. 1C and 3C). However, few acinar cells distributed homogeneously throughout the pancreas were X-Gal stained. Seven days after adenovirus administration, the percentage of acinar cell transduction was about 0.3% of total exocrine pancreas (Fig. 3E). No significant decrease in β-Gal-expressing acinar cells was observed on day 30 (Fig. 3E and F), which was consistent with the absence of lymphocytic infiltration (Fig. 3D). In contrast, when adenoviruses (5×10^8 PFU) were injected directly into the pancreas, increased exocrine cell transduction was observed around the area of injection (Fig. 3D). However, severe infiltration of inflammatory cells was also noted (Fig. 3D), which led to loss of β-Gal-expressing cells and may compromise organ functionality. It has been reported that from day 3 to day 7 after adenovirus injection into immunocompetent mice, expression of the foreign genes drops strongly (McClane *et al*., 1997b). Thus, although the level of transduction of exocrine pancreas is lower, systemic delivery in portal clamped animals is advantageous because the persistence of the engineered acinar cells may allow long-term expression of foreign genes. Gene transfer to acinar cells may be of interest for producing proteins that may act in the pancreas or be exported to ductal lumen or bloodstream (Goldfine *et al*., 1997; Vickers *et al*., 1997; Schmid *et al*., 1998).

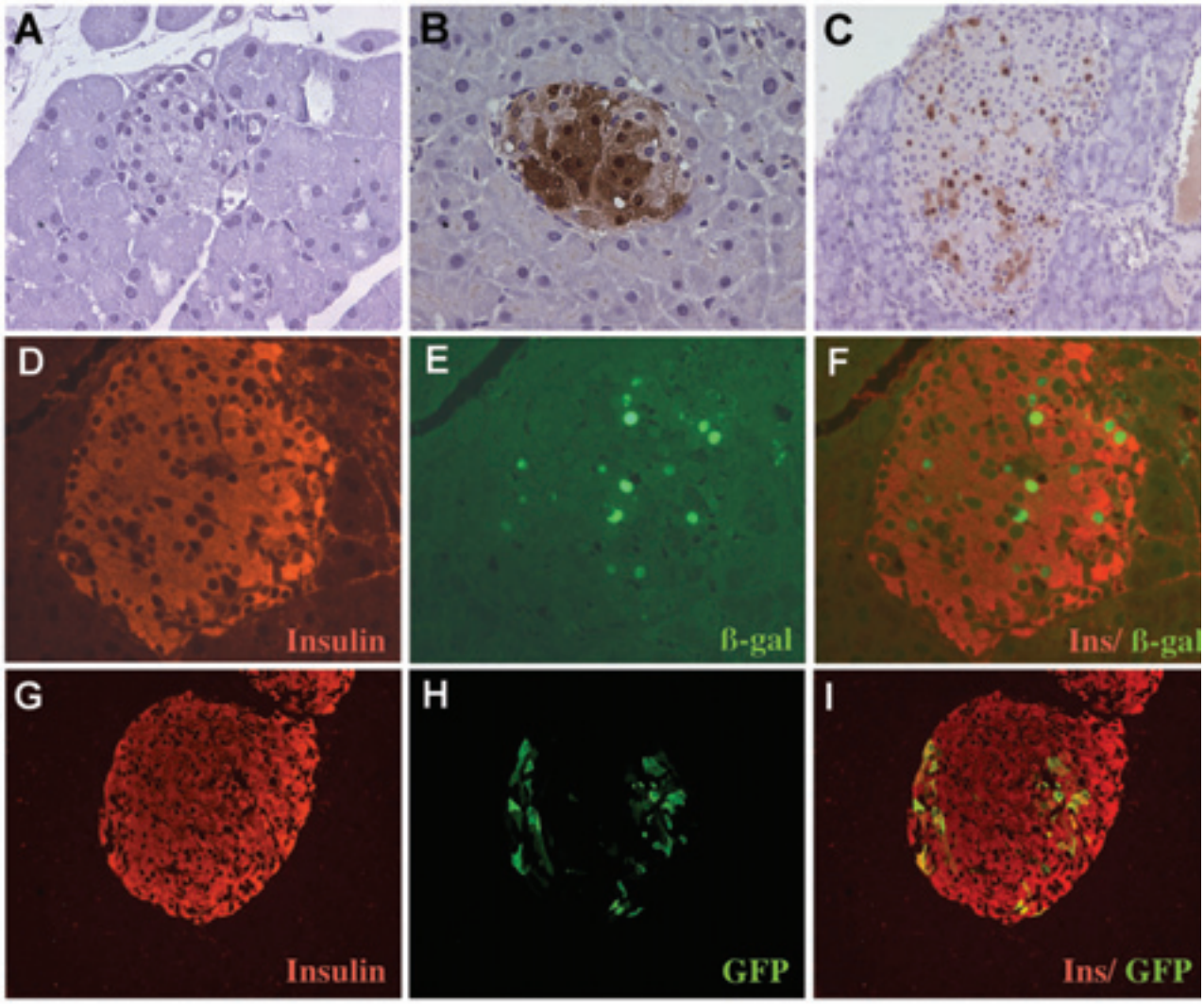
Systemic delivery of adenovirus in clamped mice infected the pancreas, but when the clamp was released the liver removed adenovirus from circulation and β-Gal expression was detected in the hepatic parenchyma (Fig. 3F). However, in contrast to exocrine pancreas, 30 days after viral injection no β-Gal-transduced cells were observed in liver (Fig. 3F). This fact may be consistent with the immune response to adenovirus-infected cells (Liu and Muruve, 2003) and also with hepatic CMV promoter silencing (Guo *et al*., 1996). To prevent expression of the gene of interest in undesired tissues, cell-type specific promoters could be used to direct the expression of the genes of interest to beta cells or acinar cells.

DISCUSSION

This study demonstrates that gene transfer to mouse beta cells *in vivo* can be achieved by systemic delivery of adenoviral vectors, bypassing the hepatic circulation. In contrast to direct pancreatic injection of adenoviral vectors, which leads to few transduced endocrine cells (Raper and DeMatteo, 1996; McClane *et al*., 1997a,b; Wang *et al*., 2004), we found that about 70% of pancreatic islets showed β-Gal expression, with an average of about 20% of the cells within positive islets being transduced. The fact that beta cells were preferentially transduced compared with acinar cells may result from both the high vascularization of islets and also the fenestrated structure of islet capillaries (Lammert *et al*., 2003). Similarly, renal glomeruli, which also contain fenestrated capillaries, are specifically transduced by systemic delivery of adenovirus in portal clamped animals (Ye *et al*., 2002).

Despite the high transduction observed in the islets 7 days after adenovirus administration, a significant decrease in β-Gal expression was observed at 14 and 30 days. It has been described that first-generation adenoviral vectors induce immune response against transduced cells, because viral proteins are expressed at low levels (Liu and Muruve, 2003). When the incidence and severity of the lymphocytic infiltration of islets were measured, we found that only a small percentage of β-Gal-expressing islets (up to 20%) presented periinsulitis, which did not progress to severe insulitis 1 month after adenoviral vector injection. To avoid undesirable effects of first-generation ade-

FIG. 2. Pancreatic islets were efficiently transduced. (**A**–**C**) β-Gal immunohistochemical detection in pancreatic sections 7 days after adenovirus administration. (**A**) No β-Gal-positive nuclei were detected in the islets of animals without portal clamp. (**B** and **C**) β-Gal-positive cells were observed within the islets of clamped animals. (**D**–**F**) Insulin (**D**) and β-Gal (**E**) double immunostaining was carried out in transduced islets. In the merged image β-Gal-positive nuclei were clearly surrounded by cytoplasmic insulin (**F**). (**G**–**I**) Insulin (**G**) and GFP (**H**) double immunostaining in transduced islets. In the merged image the yellow color documents colocalization (**I**). (**J**) Percentage of transduced islets per mouse pancreas 7, 14, and 30 days after virus administration was determined as indicated in Materials and Methods. Each point represents an animal and the bar indicates the mean value. (**K**) Percentage of transduced beta cells per islet 7, 14, and 30 days after virus administration was determined as indicated in Materials and Methods. All transduced islets from three animals were analyzed on day 7, from three animals on day 14, and from two animals on day 30. Each point represents an islet and the bar indicates the mean value. Original magnification: (**A**, **B**, and **D**–**F**) $\times$400; (**C** and **G**–**I**) $\times$200. *$p < 0.05$; **$p < 0.01$.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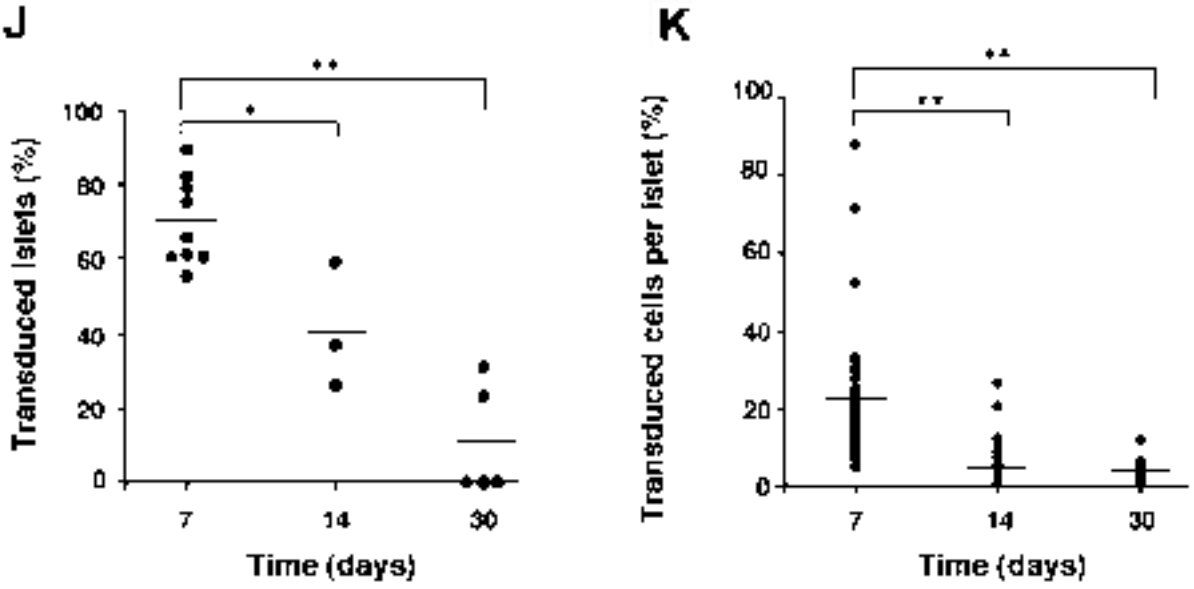
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FIG. 2.

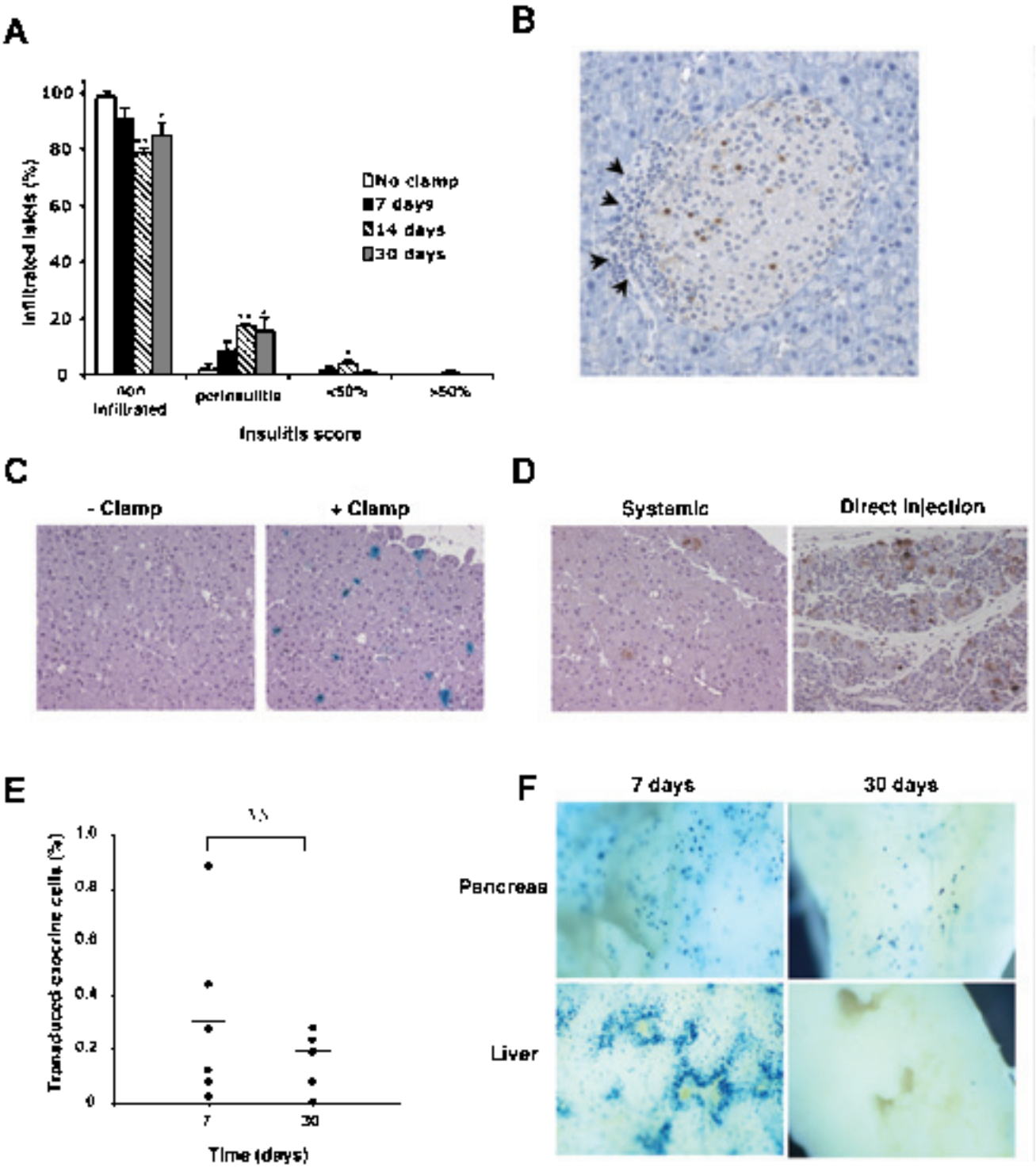

FIG. 3. Lymphocytic infiltration of islets and exocrine pancreas transduction. (**A**) Islets were scored for inflammation as described in Materials and Methods. Histograms depict percentage of noninfiltrated islets, periinsulitis, moderate insulitis (<50% of the islet infiltrated), and severe insulitis (>50% of the islet infiltrated). Lymphocytic infiltration was measured 7 days ($n = 8$), 14 days ($n = 3$), and 30 days ($n = 3$) after adenovirus injection into clamped mice, whereas nonclamped mice (No clamp, $n = 3$) were used as controls. (**B**) Representative image of periinsulitis in a β-Gal-expressing islet is shown. β-Gal-positive nuclei are brown. Arrows point to lymphocytes. Original magnification: ×400. (**C**) After *in toto* X-Gal staining, pancreas was included in paraffin, sectioned, and counterstained with hematoxylin. (−Clamp), exocrine pancreas was not transduced; (+Clamp), scattered acinar cells were transduced in the pancreas of clamped animals. Original magnification: ×200. (**D**) Lymphocytic infiltration in the exocrine pancreas 7 days after virus administration either by systemic delivery or by direct injection. β-Gal immunostaining (brown) in pancreatic sections demonstrates transduced cells. Direct injection of adenoviruses into the pancreas led to increased β-Gal-expressing cells, but severe inflammation was observed. Original magnification: ×200. (**E**) Percentage of transduced exocrine cells 7 and 30 days after virus administration was determined as indicated in Materials and Methods. Each point represents an animal and the horizontal bars indicate mean values. NS, not significant ($p = 0.45$). (**F**) *In toto* X-Gal staining showed persistence of β-Gal in the pancreas, but not in the liver, 30 days after vector administration. Original magnification: pancreas, ×25; liver, ×35.

noviral vectors that lead to transient expression of the genes of interest, helper-dependent adenoviral (HDAd) vectors may be used (Kay *et al*., 2001). These vectors minimize the host adaptive response and improve the efficacy and duration of gene transfer *in vivo* (Lieber *et al*., 1996; Parks *et al*., 1996; Morral *et al*., 1999). In addition to viral proteins, β-Gal may have increased the immune response against transduced cells. Expression of β-Gal in the skeletal muscle of immunocompetent mice, using HDAd vectors, resulted in a decrease in vector copy number as early as 28 days after injection, and complete loss of β-Gal expression in two of three mice at 42 days (Chen *et al*., 1997). These mice showed extensive inflammatory cellular infiltration consisting primarily of $CD4^+$ and $CD8^+$ lymphocytes, associated with the expression of β-Gal (Chen *et al*., 1997). Similarly, loss of expression was also observed when using HDAd β-Gal vectors in lung (Toietta *et al*., 2003). Furthermore, because β-Gal expression in our experiments is under the control of the CMV promoter, immune-mediated repression of the CMV promoter cannot be ruled out. It has been shown that interferon γ, which would be produced in the course of T cell-mediated immune response, can inhibit the CMV promoter (Harms and Splitter, 1995).

In addition to adenovirus, lentiviral and adeno-associated viral (AAV) vectors can infect islets *in vitro* (Giannoukakis *et al*., 1999a; Flotte *et al*., 2001) and may be used to transduce endocrine pancreas *in vivo* for long-term expression of genes of interest. *In vitro* studies showed that lymphocytic choriomeningitis virus-pseudotyped lentiviral vectors transduce insulin-secreting beta cells with greater efficiency than lentiviral vectors pseudotyped with vesicular stomatitis virus, murine leukemia virus, Ebola, rabies, or Mokola (Kobinger *et al*., 2004). Furthermore, AAV-mediated gene transfer to murine islets *in vitro*, using nonserotype 2 AAV capsids, can mediate more efficient transduction of islet cells, with AAV1 being the most efficient serotype (Loiler *et al*., 2003). It has been shown that AAV8 is more efficient than AAV2 in transducing the pancreas *in vivo* after direct injection, whereas AAV5 does not result in any detectable transgene expression (Wang *et al*., 2004). Nevertheless, this study also shows that adenoviral vectors are more efficient than AAV8 in transducing the pancreas *in vivo*. Persistence of gene expression was longer with AAV vectors than with adenoviral vectors, which elicit significant leukocyte infiltration and greater than 90% of expression lost after 4 weeks (Wang *et al*., 2004).

In summary, our study showed that systemic injection of adenovirus into mice with clamped hepatic circulation was an efficient approach to deliver genes of interest to mouse pancreatic beta cells *in vivo*. Thus, several factors involved in pancreas regeneration (George *et al*., 2002), beta cell differentiation (Kojima *et al*., 2003), and beta cell preservation and function (Giannoukakis *et al*., 1999b; Grey *et al*., 1999; Heimberg *et al*., 2001) may be expressed in mouse pancreatic islets *in vivo* by this approach. However, in large animals and humans, to increase pancreas transduction and to avoid liver damage after portal clamp, a local pancreatic circulation clamp might be performed. Thus, progress in diabetes therapy by transferring key therapeutic genes should be possible in the near future. In addition, this methodology may be used to study islet biology and also new therapeutic strategies for other pancreatic disorders.

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IX. ANNEX II

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ARTICLE

In Vivo Gene Transfer to Healthy and Diabetic Canine Pancreas

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Gene therapy may provide new treatments for severe pancreatic disorders. However, gene transfer to the pancreas is difficult because of its anatomic location and structure, and pancreatitis is a serious concern. Like the human pancreas, the canine pancreas is compact, with similar vascularization and lobular structure. It is therefore a suitable model in which to assess gene transfer strategies. Here we examined the ability of adenoviral vectors to transfer genes into the pancreas of dogs in which pancreatic circulation had been clamped. Adenoviruses carrying the β-galactosidase (β-gal) gene were injected into the pancreatic–duodenal vein and the clamp was released 10 min later. These dogs showed β-gal-positive cells throughout the pancreas, with no evidence of pancreatic damage. β-Gal was expressed mainly in acinar cells, but also in ducts and islets. Moreover, transduction was prominent in connective tissue of the lobe septa. β-Gal expression in the exocrine pancreas of a diabetic dog was also found to be similar to that observed in healthy dogs. Thus, efficient gene transfer to canine pancreas *in vivo* may be achieved by adenovirus injection after clamping pancreatic circulation. This technique may be used to assay new gene therapy approaches for diabetes mellitus and other pancreatic disorders.

Key Words: pancreas, dog, adenoviral vectors, gene transfer, diabetes

INTRODUCTION

Beagle dogs have been used as large animal models of many human diseases, and several gene therapy approaches, such as strategies for hemophilia and retinal degeneration, have been assayed [1–5]. However, gene transfer to canine pancreas has not yet been reported. Genetic manipulation of this organ requires thorough knowledge of its anatomy, especially the vascularization, since there are major differences compared to the rodent pancreas. It is located in the dorsal part of the abdominal cavity close to the proximal part of the duodenum. In the dog, it has the classic V shape, consisting of two lobes (right and left) that emerge from the pancreatic body, surrounded by a delicate capsule of connective tissue. Septa from the capsule divide the pancreas into lobules, delimited by connective tissue, which produce a nodular surface with irregular crenate margins. Between the lobules, connective tissue surrounds the larger ducts, blood vessels, and nerve fibers. Branches from the celiac and the cranial mesenteric arteries supply blood to the pancreas. The pancreatic branches of the splenic artery irrigate the left lobe, whereas the right lobe is irrigated by the cranial and caudal pancreaticoduodenal arteries (branches from the gastroduodenal artery (cranial) and cranial mesenteric artery (caudal), respectively) (Fig. 1A). Anastomoses are common in pancreatic circulation. Veins are parallel to the arteries and eventually drain into the portal vein (Fig. 1A).

In type 1 and type 2 diabetes, hyperglycemia develops when pancreatic insulin secretion fails, as a result of β-cell loss. Gene transfer to the pancreas to induce β-cell regeneration from islet cell precursors *in vivo* may revert these diseases. Successful genetic engineering of the pancreas *in vivo* will depend on the appropriate choice of both the route of adminis-

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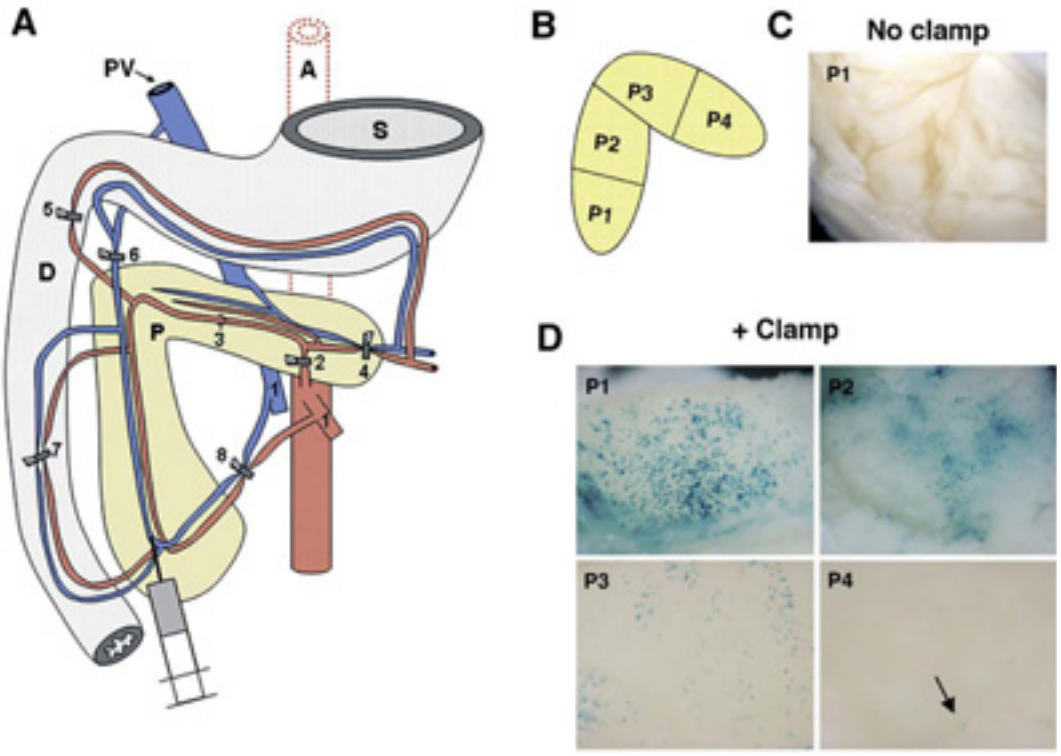

FIG. 1. (A) Scheme of canine pancreatic circulation showing the clamping sites. Stomach (S), duodenum (D), pancreas (P), aorta (A), and portal vein (PV) are indicated. The arterial system is colored in red and venous system in blue. 1, Cranial mesenteric artery and vein. 2, Celiac artery. 3, Hepatic artery. 4, Splenic artery and vein. 5, Right gastroepiploic artery. 6, Gastroduodenal vein. 7, Cranial pancreaticoduodenal artery and vein. 8, Caudal pancreaticoduodenal artery and vein. The syringe shows the site of virus injection. (B–D) Pancreas gene transfer of dogs that underwent pancreatic circulation clamp. (B) Pancreas from clamped dogs were removed 5 days after adenoviral vector administration and divided into four parts, P1, P2, P3, and P4, as indicated. (C) No X-gal staining was detected in pancreas of nonclamped dogs after *in toto* analysis. (D) X-gal staining of the pancreas revealed that adenoviral transduction was extended mainly from P1 to P3, although a few transduced cells in P4 (arrow) were also observed. Original magnification 25×.

tration and the vector. In rodents, systemic delivery of adenovirus does not infect the pancreas *in vivo,* because the liver rapidly clears the virus from circulation [6]. However, we have shown that systemic injection of adenovirus after a temporary closure of the portal vein, hepatic artery, and bile duct (portal clamp) results in increased concentration of circulating virus during the clamp and in transduction of both the exocrine and endocrine pancreas [7]. An approach of this kind is difficult to apply in large animals, since liver ischemia could result, and adenoviral vectors could be toxic when injected systemically in large amounts [8,9]. However, taking into account the anatomy and vascularization of the dog pancreas, a clamp of pancreatic circulation may be applied *in situ* and vectors can be injected directly into the pancreatic vessels. This may increase pancreas transduction and avoid liver damage. Several vectors have been used to transfer foreign genes to pancreatic islets and pancreatic β-cell lines *in vitro* [10–21]. Among them, adenovirus shows β-cell tropism and high transduction efficiency both *in vitro* [13,15,17,18] and *in vivo* [7].

Since gene transfer to canine pancreas *in vivo* is difficult because of its anatomic location and structure, we performed a local clamp of pancreatic circulation, followed by *in situ* injection of adenoviral vectors. This strategy led to the successful transfer of the β-galactosidase (β-gal) gene to the exocrine and endocrine pancreas. Surgery and vector administration were also performed in a diabetic dog, and exocrine pancreas β-gal expression was similar to that of healthy dogs. Thus, this methodology may be used to assay new gene therapy approaches for diabetes mellitus.

RESULTS

Adenovirus-Mediated Gene Transfer to Pancreas was Achieved in Dogs with Clamped Pancreatic Circulation

Since blood supply to the pancreas comes from multiple vessels, we established clamps at several sites to achieve blood stasis. We clamped the following vessels before vector administration: celiac, splenic, gastroduodenal, gastroepiploic, and cranial and caudal pancreaticoduode-

nal arteries. As the veins parallel the arteries, clamps occluded both types of vessel (Fig. 1A). Once the clamp was established, we infused a solution containing adenoviral vectors (2×10^{10} IU (infection units)/dog) carrying the β-galactosidase marker gene under the control of the CMV promoter (AdCMV/β-gal) into the pancreaticoduodenal vein (Fig. 1A).

We euthanized the dogs 5 days later. To study the distribution of the β-gal-expressing cells we sectioned the pancreas into four pieces, P1, P2, P3, and P4 (Fig. 1B). Five days after vector injection into the pancreatic–duodenal vein, β-gal was expressed throughout the pancreas (Fig 1D). However, β-gal expression was higher in P1 and P2, which were closer to the site of vector injection. Transduction was lower in P3 and much lower in P4, in which only a few cells were β-gal positive (Fig 1D). Thus, while efficient transduction was achieved when viruses were injected through the pancreatic–duodenal vein in clamped dog pancreas, in nonclamped pancreas transduction was not observed (Figs. 1C, 2A, and 2D). We have quantified the percentage of positive cells in histological sections stained for β-gal. In the whole pancreas 1.7 ± 0.7% of cells were transduced (P1, 3.3 ± 1.7%; P2, 1.7 ± 1; P3, 0.3 ± 0.2%; and P4, 0.01 ± 0.008%; $n = 3$).

β-Gal expression after injection into the pancreaticoduodenal artery was similar to that observed following injection into the vein (Fig. 2). These results suggest that both routes of administration, vein and artery, may be used when blood stasis is established within the pancreas.

Cell Types Transduced by Adenovirus in Canine Pancreas

β-Gal-positive cells were located near the lobe septum (Fig. 3A), especially in the connective tissue forming the septa (Figs. 3B and 3C). Furthermore, acinar cells showed prominent β-gal expression (Fig. 3E). In addition, we detected positive nuclei for β-gal staining in ducts of clamped animals (Fig. 3G). After both venous and arterial delivery of the vectors we observed that acinar cells were preferentially transduced. Neither acinar nor ductal cells expressed β-gal in dogs that were not clamped (Fig. 3D and 3F, respectively).

We also analyzed the distribution and shape of dog islets by insulin and glucagon immunostaining of pancreatic sections (Figs. 4A–4F). Canine pancreas contained large number of small islets homogeneously distributed throughout the section (Fig. 4D). The mouse pancreas, in contrast, contained significantly fewer islets (dogs, 11.7 ± 1.1 islets/mm^2 of pancreas, vs mice, 1.47 ± 0.1 islets/mm^2 of pancreas, $P < 0.05$), although they were larger (mouse islet area, 3878 ± 190 μm^2, vs dog, 1075 ± 96 μm^2, $P < 0.05$) (Fig. 4A). In mouse islets, glucagon-producing α cells were distributed mainly at the periphery, whereas insulin-producing β cells were seen in the core (Figs. 4B and 4C). In contrast, dog islets showed irregular α-and β-cell distribution (Figs. 4E and 4F). Furthermore, we observed scattered endocrine cells, either α or β cells, throughout the dog pancreas (Figs. 4D–4F). Double immunostaining revealed cells in the islets that were positive for insulin and β-gal (Fig. 4G). Furthermore, we also detected many β-gal-positive cells around the islets, suggesting that adenovirus transduction of dog pancreas was not selective for a specific cell type.

When we released the clamp adenovirus escaped from the pancreatic circulation, into the general circulation, and was taken up by the liver. However, β-gal expression in the liver was similarly low in clamped and nonclamped animals (Fig. 4H).

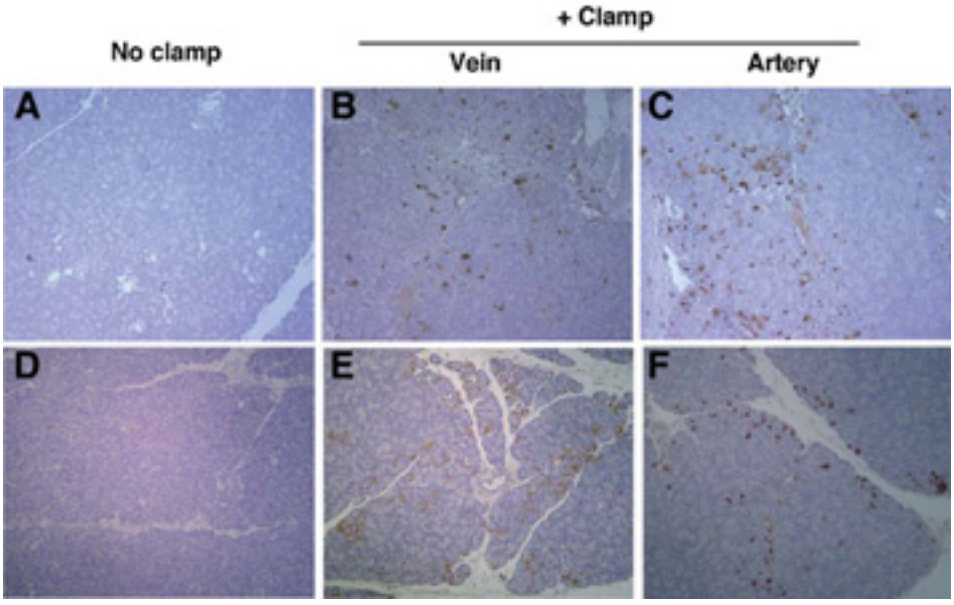

FIG. 2. Immunohistochemical analysis of β-gal expression in the pancreas. (A, D) Nonclamped and (B, C, E, F) clamped dog pancreas were analyzed 5 days after vector administration. (A, D) No β-gal expression was observed in nonclamped animals. (B, C, E, F) Pancreas gene transfer was achieved in clamped dogs when the injection of the adenovirus was performed either by the vein (B, E) or by the artery (C, F). Original magnification 100×.

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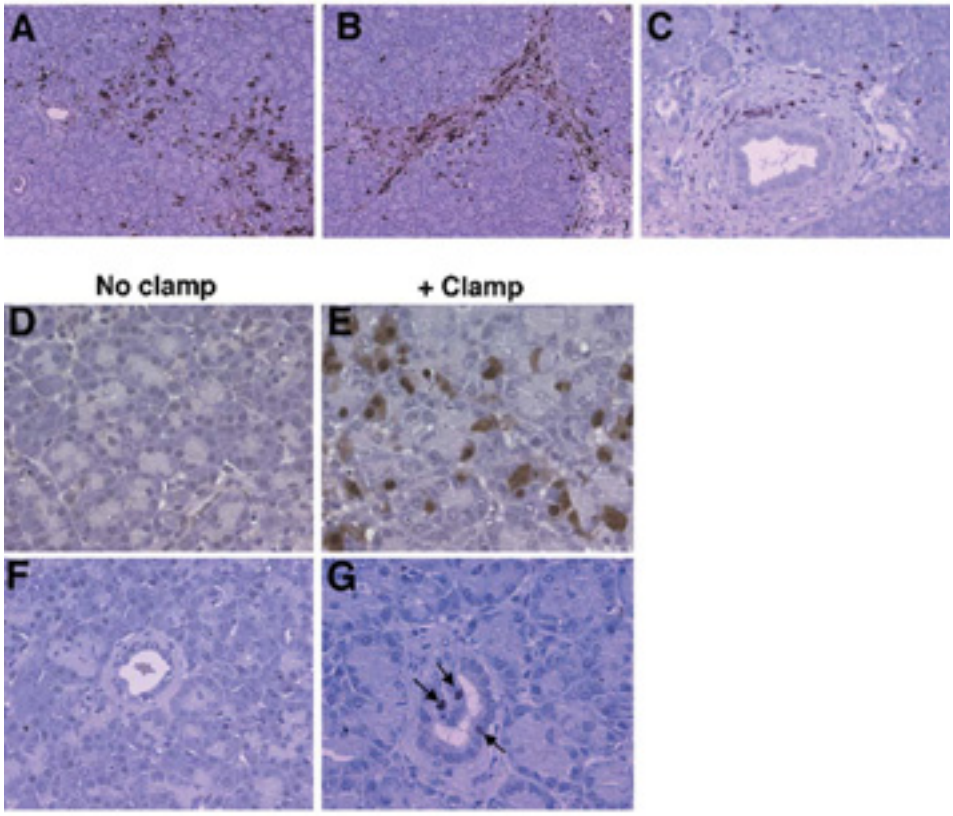

FIG. 3. (A–C) Connective tissue in the septa was efficiently transduced. Septa divide the pancreas into lobules, bounded by connective tissue. Between the lobules, connective tissue surrounds the larger ducts, blood vessels, and nerve fibers. (A) β-Gal-positive cells located around the septa can be observed (original magnification 100×). (B) Adenovirus transduction of the connective tissue in a longitudinal section (original magnification 100×). (C) β-Gal-positive cells in the connective tissue surrounding pancreatic duct in a cross section (original magnification 200×). (D–G) Gene transfer to acinar and ductal cells of dog pancreas. β-Gal immunostaining was performed in pancreatic sections 5 days after adenovirus injection. (E) Acinar cells were highly transduced by the adenovirus in clamped dog. (D) In contrast, exocrine pancreas was not transduced in dogs without clamp. (G) Ductal cells were also transduced by the adenovirus in animals that underwent pancreatic clamp. Original magnification 400×.

To evaluate pancreas and liver damage caused by the clamp and/or the adenoviral vectors, serum samples were taken the day before surgery (control value) and 1, 2, 5, and 15 days thereafter. Amylase and lipase are common markers of pancreatic damage, and alanine-amino transferase (ALT) is a marker of liver damage. ALT, amylase, and lipase levels were within the normal range 5 days after surgery (Table 1). Only one of the 11 dogs that were clamped showed amylase and lipase levels above normal values 5 days after the surgery. However, we observed no symptoms of severe pancreatitis, such as vomiting or diarrhea.

Gene Transfer to the Pancreas of a Diabetic Dog

We treated one dog with a single intravenous injection of a streptozotocin (STZ) and alloxan mixture to induce experimental diabetes [22]. Since destruction of β cells led to massive insulin release a few hours after the STZ/alloxan injection, the dog developed hypoglycemia. To maintain normoglycemia during this period, we monitored the dog and controlled glycemia by glucose infusion. Two days after STZ/alloxan injection, we detected hyperglycemia (>250 mg/dl) and maintained blood glucose levels below 300 mg/dl by subcutaneous injections of 8 IU of soluble insulin (Fig. 5A). Afterward, we carried out surgery on the diabetic dog, clamped the pancreatic circulation, and injected 2×10^{10} IU of AdCMV/β-gal into the pancreaticoduodenal vein. Insulin immunostaining confirmed the loss of β cells in the diabetic pancreas (Figs. 5B and 5C). This was consistent with the decrease in serum insulin levels (data not shown). β-Gal was expressed throughout the pancreas in the diabetic dog (Fig. 5E), mainly in the acinar cells and similar to healthy dogs (Fig. 5D). Thus, these results indicate that surgery and vector administration were feasible under diabetic conditions and that the pancreas was efficiently transduced.

DISCUSSION

Successful genetic engineering of the pancreas *in vivo* will depend on the appropriate choice of both the route of administration and the vector. Large animals are good models of human diseases, especially the dog, but canine pancreas and murine pancreas are different. Adenoviruses show high efficiency in infecting islets *in vitro* [13,15, 17,18] as well as mouse exocrine and endocrine pancreas *in vivo* [7,23–30]. In mice, direct pancreatic injection of adenoviruses leads to high transduction of exocrine pancreas around the site of injection, but only a few endocrine cells in the periphery of islets are infected [28]. In contrast, in mice with clamped hepatic circulation, systemic injection of adenoviral vectors leads to transduction of most islets, and scattered acinar cells express-

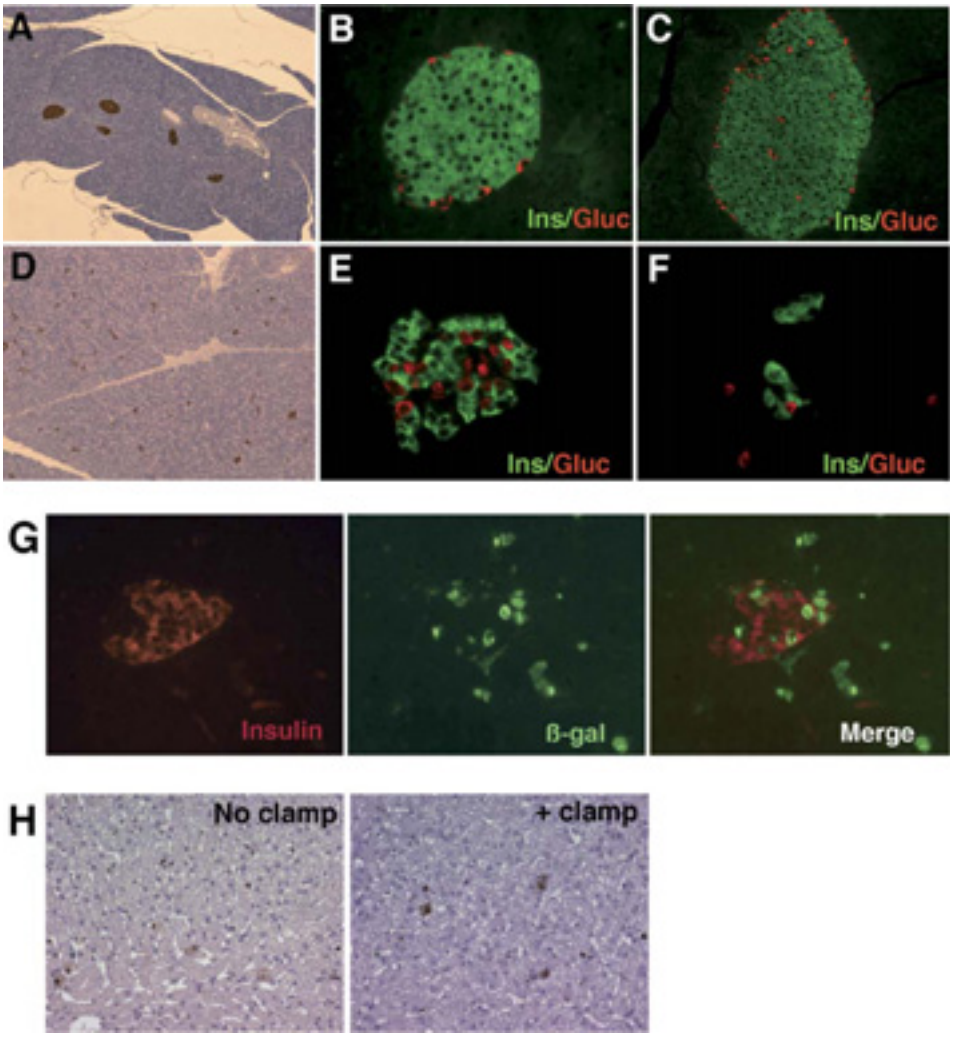

FIG. 4. Analysis of dog endocrine pancreas. (A–F) The distribution of insulin-producing cells in canine islets was determined in paraffin sections and compared with that of mouse islets. Mouse pancreas (A) showed a small number of larger islets. Islet architecture was examined by double immunostaining of insulin (green) and glucagon (red). In mouse islets, β cells reside in the core, whereas the α cells are located in the periphery (B, C). Altered distribution of β and α cells was observed in canine islets (E, F). Furthermore, small groups of α and β cells distributed throughout the pancreas can also be observed (F). Original magnifications 40× (A, D), 200× (C), and 400× (B, E, F). (G) Endocrine pancreas transduction. Insulin and β-gal double immunostaining was carried out in transduced pancreas. β cells expressing β-gal can be observed (original magnification 400×). (H) Liver transduction. Transduction level was similar in clamped and nonclamped animals. Equal amounts of viral vectors were used. β-Gal immunostaining of liver sections is shown. Original magnification 200×.

ing β-gal are also observed throughout the pancreas [7]. Thus, intravascular delivery of adenoviral vectors allows transduction of larger areas of the pancreas than direct injection, which can also induce severe inflammation and toxicity [26–28]. In the present study in dogs, injection of adenoviral vectors into the pancreatic circulation led to efficient *in vivo* gene transfer throughout the pancreas. Most of the β-gal immunostaining was seen in acinar cells and in connective tissue surrounding the main blood vessels and in the islets and ducts. However, after intravascular injection, the pattern of transduction differed from that seen in mice, in which β cells are preferentially transduced rather than acinar cells, because of the high vascularization of islets and the fenestrated structure of islet capillaries [31]. The presence of endocrine cells scattered throughout the pancreas and morphological differences in canine islets might explain these discrepancies. Furthermore, the local clamp induced blood stasis in the dog pancreas, so distribution of the adenoviruses was limited. In addition, dogs and rodents also differ in their lobe structure and blood supply. In dogs, connective tissue surrounds the blood vessels, ducts, and nerve fibers, as in the human pancreas. Since vectors were injected intravascularly, transduction was high in connective tissue and acinar cells and pancreatic islets near the septa showed higher β-gal expression than those located in the center of the lobe.

Dogs were euthanized 5 days after adenoviral vector administration, when maximal expression of the delivered gene was expected. Long-term expression cannot be expected using the β-gal reporter gene and first-generation adenoviruses. These vectors induce an immune response against transduced cells, since viral proteins are expressed at low levels [32]. Furthermore, β-gal can also induce an immune response, since it is a foreign protein, in which case transduced cells are eliminated [33,34]. To avoid undesirable effects of first-generation adenoviral vectors that lead to transient

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TABLE 1: Serum enzyme activity after surgery

	ALT (U/L)				Amylase (U/L)				Lipase (U/L)			
	D 0	D 1	D 5	D 15	D 0	D 1	D 5	D 15	D 0	D 1	D 5	D 15
Dog 1	26	29	96	—	1162	1248	1173	—	111	134	72	—
Dog 2	41	36	75	—	1129	825	1166	—	87	21	76	—
Dog 3	96	65	61	—	1173	1471	1948	—	72	72	83	—
Dog 4	63	—	31	—	832	—	1170	—	196	—	126	—
Dog 5	39	41	101	—	690	839	501	—	87	277	265	—
Dog 6	87	92	78	—	1063	849	850	—	257	93	76	—
Dog 7	25	—	29	—	1096	—	1299	—	225	—	216	—
Dog 8	44	—	1395[a]	—	1041	—	4000[a]	—	111	—	6628[a]	—
Dog 9	34	—	25	27	938	—	1057	911	81	—	53	52
Dog 10	34	—	36	29	704	—	554	600	57	—	48	41
Dog 11 (nonclamped)	24	—	23	—	637	—	713	—	107	—	62	—
Dog 12 (diabetic)	—	—	—	—	1385	1299[b]	—	—	263	211[b]	—	—

Serum samples were taken the day before surgery (control value) and 1, 2, 5, and 15 days thereafter. Normal values: ALT, 21–102 U/L; amylase, 185–2000 U/L; lipase, 13–200 U/L. Dogs 1 to 10 were healthy dogs that underwent pancreatic clamp. Dog 11 was unclamped. Dogs 1–8 were injected with 2×10^{10} IU of adenoviral vectors. Dogs 9 and 10 were injected with saline. Dog 12 was a diabetic dog that underwent pancreatic clamp. —, not determined.

[a] Parameter out of the upper range.

[b] Serum value of day 2 instead of day 1 after the surgery.

expression of the gene of interest, helper-dependent adenoviral vectors may be used [35–37]. These vectors minimize the host adaptive response and improve the efficacy and duration of gene transfer *in vivo* [1,38–43]. In addition to adenoviruses, adenoassociated viruses (AAV) can be used for pancreas gene transfer *in vivo* [23]. It has been shown that AAV8 is more efficient than AAV2 in transducing mouse pancreas after direct injection, whereas AAV5 injection does not lead to any detectable transgene expression [23]. Nevertheless, the study of Wang *et al.* also shows that adenoviral vectors are more efficient than AAV8 for engineering pancreas *in vivo* [23]. It has been recently reported that AAV8 vectors injected into mouse pancreatic duct led to efficient transduction of acinar cells with less than 5% of ductal cells being transduced and a minimal amount of β cells transduced [44]. However, AAV1 serotype was more efficient than AAV8 in rat pancreas [44].

In addition to pancreas transduction we also detected liver transduction. To prevent expression of the gene of interest in undesired tissues, cell-type-specific promoters could be used to direct the expression of the genes of interest to β cells or acinar cells. In addition, the use of tissue-specific promoters reduces the immune response against the transgene when adenoviral vectors are used [45].

Several studies have shown that β-cell precursors reside both in pancreatic ducts and inside mouse islets [46–49]. Since we observed transduced cells in ducts and inside the islets, genes involved in pancreas regeneration, β-cell differentiation, β-cell preservation, and function may be expressed in canine pancreas *in vivo* using this approach. Furthermore, acinar cells were highly transduced and they might be used to express and secrete proteins that may act in the surrounding islets in a paracrine manner. Thus, progress in diabetes therapy by transferring key genes directly to the pancreas in large animals should be possible and may lead to the development of treatment for humans. In addition, this methodology may be used to study new therapeutic strategies for other pancreatic disorders.

MATERIALS AND METHODS

Animals. Male beagle dogs, 6–12-months of age, were used (Isoquimen, Barcelona, Spain). They were fed once a day with a standard diet and kept under a natural light cycle. Male C57BL/6SJL mice, 2 months of age, were used (CBATEG, Barcelona, Spain). Animal care and experimental procedures were approved by the Ethics Committee on Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

Recombinant adenoviral vector. Human E1-deleted recombinant serotype 2 adenoviruses carrying the cytomegalovirus promoter/β-galactosidase chimeric gene (AdCMV/β-gal) were generated as described previously [50]. Infectious units were determined by infecting 293 cells with serial dilutions of the virus and then counting β-gal-expressing cells after 48 h. The β-galactosidase contains a nuclear localization signal. The particle/IU ratio of the virus stock used in the experiments was between 10 and 40.

Local pancreatic clamp and administration of adenovirus. All dogs received an im injection of a neuroleptoanalgesic combination of acepromazine (0.05 mg/kg) and buprenorfine (0.01 mg/kg). Thirty minutes after the preanesthetic medication, anesthesia was induced by intravenous injection of 4 mg/kg propofol and maintained with 1–2% isoflurane in oxygen. Following laparotomy, pancreatic circulation was clamped (including celiac, splenic, gastroduodenal, gastroepiploic, and cranial and caudal pancreaticoduodenal arteries). Blood vessels were occluded with hemostatic clamps. Since pancreas and duodenum share a common blood supply we also clamped the duodenum by plastic-protected intestinal clamps. Afterward, 2 ml of virus suspension (in 150 mM NaCl) was injected into the pancreaticoduodenal vein (or artery). A total viral dose of 2×10^{10} IU per animal was used. Ten minutes later, the clamp was removed and the abdominal wall was sutured. Surgery and

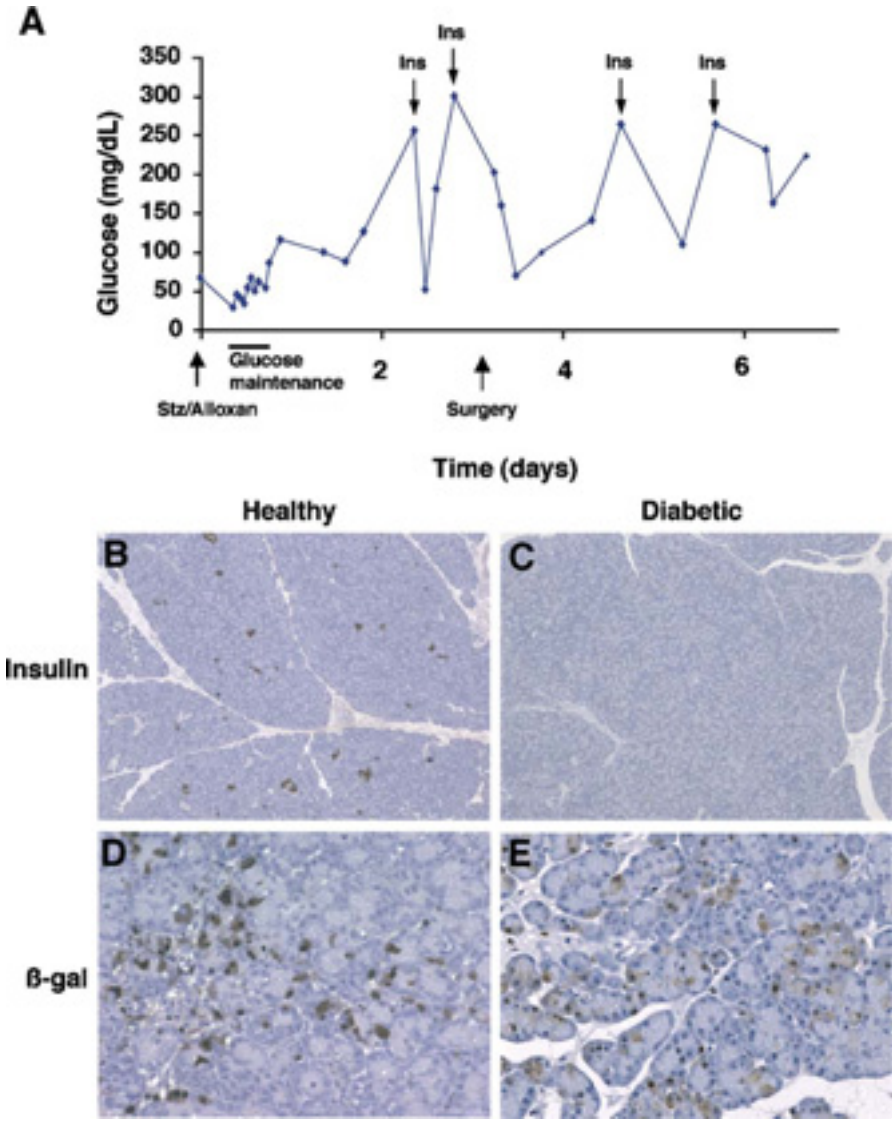

FIG. 5. Gene transfer to diabetic canine pancreas. (A) Experimental diabetes was induced by streptozotocin/alloxan injection as described under Materials and Methods. A few hours afterward, the animal developed hypoglycemia due to massive destruction of β cells and insulin release. Glycemia was controlled by glucose infusion. When hyperglycemia was observed, the dog was treated with subcutaneous injection of 8 IU of soluble insulin (Ins). Surgery and vector administration were performed 3 days after diabetes induction. (B, C) Reduction of β-cell mass was observed in the diabetic dog after insulin immunostaining of pancreatic sections. (D, E) Gene transfer was also achieved in diabetic pancreas. Pancreatic sections of a healthy (D) and a diabetic (E) dog immunostained with β-gal antibody (brown) are shown. Original magnifications 40× (A, B) and 200× (D, E).

vector dose (injected into the pancreaticoduodenal vein) used in unclamped dog were the same as in clamped; however, blood vessels were not occluded. Five days after vector administration, animals were euthanized with intravenous injection of pentobarbital overdose, and the pancreas and liver were removed. Experimental diabetes was induced in one dog by a single intravenous injection (by cephalic vein) of an STZ (35 mg/kg body wt) and alloxan (40 mg/kg body wt) mixture [22].

Analysis of β-galactosidase expression in tissue samples. To detect the presence of β-galactosidase in pancreas and liver *in toto,* samples were fixed for 1 h in 4% paraformaldehyde, washed twice in phosphate-buffered saline (PBS), and then incubated in X-gal (5-bromo-4-chloro-3-β-D-galactopyranoside) in 5 mM $K_3Fe(CN)_5$, 5 mM $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in PBS for 6–8 h in the dark at 37°C.

Immunohistochemical and morphometrical analysis. For immunohistochemical detection of β-galactosidase, insulin, and glucagon, dog and mouse pancreas were fixed for 12 to 24 h in formalin, embedded in paraffin, and sectioned. Sections were then incubated overnight at 4°C with rabbit anti-β-galactosidase antibody ab616 (Abcam, Cambridge, UK) diluted at 1:900, with a guinea pig anti-porcine insulin antibody (DAKO Corp., Carpinteria, CA, USA) at 1:100 dilution, or with a rabbit anti-human glucagon antibody (ICN Biomedicals, Inc., Cleveland, OH, USA) at 1:4000 dilution. As secondary antibody rabbit anti-guinea pig immunoglobulin G, coupled to peroxidase (Roche Molecular Biochemicals), or biotinylated goat anti-rabbit antibody and ABC complex (Vector, Burlingame, CA, USA) was used. 3′,3′-Diaminobenzidine was used as the substrate chromogen. Sections were counterstained in Mayer's hematoxylin. For immunofluorescence, FITC-labeled goat anti-rabbit (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) or TRITC-conjugated rabbit anti-guinea pig (Sigma Chemical Co.) was used as secondary antibody. For morphometrical analysis of the islets, pancreas was obtained and immunohistochemical detection of insulin was performed on four (2 to 3 μm) sections per animal (n = 3 dogs and n = 6 mice). The total number of islets/mm^2 was calculated by dividing the total number of islets in one section by the total area of this section. The area (mm^2) of each section was determined using a Nikon Eclipse E800 microscope (Nikon Corp., Tokyo, Japan) connected to a video camera with a color monitor and to an image analyzer (analySIS 3.0; Soft Imaging System Corp., Lakewood, CO, USA). The area of the islets (mm^2) was measured using the same software, and more than 500

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islets were counted both in dogs and in mice. Quantification of pancreatic cell transduction was performed in histological sections immunostained for β-gal. The percentage of transduction was obtained counting all positive cells in 20 microscopy fields in P1–P4 (40× magnification).

Determination of serum parameters. Before and after the surgery blood samples were taken for biochemical analysis. Blood glucose levels were measured with a Glucometer Elite analyzer (Bayer AG, Leverkusen, Germany). Determination of serum ALT, amylase, and lipase levels were determined spectrophotometrically using specific kits from ABX Diagnostics (Montpellier, France).

Statistical analysis. All values are expressed as the means ± SEM. Differences between groups were compared by the Student *t* test. *P* values less than 0.05 were considered statistically significant.

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